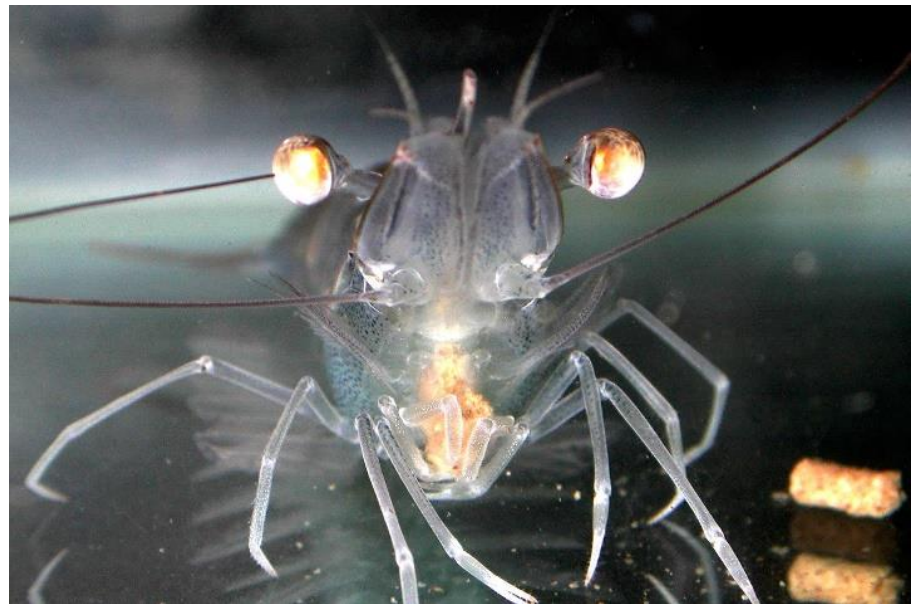


Investigación y Desarrollo en Nutrición Acuícola



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Índice

<i>Insights in to the potential Pre-processing of ingredients to improve their nutritive value to aquaculture species</i>	1
Dominique P. Bureau*, Yuhong Yang, Chunfang Cai and Guillaume Pfeuti University of Guelph, Canadá	
<i>Fact or fiction: Methionine requirement for Pacific white shrimp Litopenaeus vannamei</i>	32
Allen Davis* and Mingming Duan Auburn University, USA	
<i>El ciclo diario de la digestión en peces cultivados. Aspectos funcionales y metodológicos</i>	55
Manuel Yúfera Instituto de Ciencias Marinas de Andalucía, España	
<i>Aditivos alimentarios para camarones marinos: salud y nutrición</i>	78
Felipe Vieira*, Norha Constanza Bolivar, Esmeralda Chamorro Legarda, Delano Dias Schleder, Walter Quadros Seiffert, Leila Hayashi Universidad Federal de Santa Catarina, Brasil	
<i>La nutrición y alimentación del pepino de mar (Echinodermata, Holothuroidea); situación actual y perspectivas para el desarrollo de su cultivo intensivo</i>	106
Miguel Ángel Olvera Novoa*, Gloria Martínez-Milián y Itzel A. Sánchez-Tapia Centro de Investigación y Estudios Avanzados IPN-Mérida, México	
<i>Culture of marine sciaenids in low salinity: an opportunity for expanded aquaculture in Mexico</i>	156
Mayra L. González Félix*, Martin Perez-Velazquez, Germán E. Ibarra-Garciaparra, Jorge Trujillo-Villalba Universidad de Sonora, México	
<i>Lípidos alternativos en la nutrición de peces marinos</i>	171
Artur Rombenso Universidad Autónoma de Baja California, México	
<i>Transcriptómica la nueva puerta para el estudio de la nutrición en acuicultura: actividad enzimática en el cultivo larvario de crustáceos</i>	180
Gloria Helena Ospina Salazar*, Anselmo Miranda-Baeza, Juan F. Alzate, Sven Zea Universidad Nacional de Colombia	
<i>Uso de Microorganismos Inmovilizados en Cultivos Camaronícolas y su Efecto en la Respuesta Productiva, Condición Fisiológica y Microbioma Intestinal</i>	195
Luis Rafael Martínez Córdova*, Marcel Martínez Porchas; Anselmo Miranda Baeza; Roberto Vázquez Euan; Kadiya Calderón Alvarado, Ana Lucía Gómez Ramírez Universidad de Sonora, México	
<i>La biomasa microbiana como ingrediente en la nutrición acuícola</i>	213
Julián Gamboa Delgado*, Angel Gabriel Alvarado Ibarra, Yonatan Izahi Morales Navarro Martha G. Nieto-López, David Villarreal-Cavazos, Maribel Maldonado-Muñiz Mireya Tapia-Salazar, Denis Ricque-Marie, Lucía Elizabeth Cruz-Suárez Universidad Autónoma de Nuevo León, México	
<i>Taurine synthesis in teleosts-importance of cysteamine pathway</i>	264
Yutaka Haga*, Maria Mojena Gonzales, Tomoko Itoh, Hidehiro Kondo, Ikuo Hirono, Shuichi Satoh Universiy of Tokyo, México	

<i>Development of antibiofilm biosurfactants from marine bacteria against shrimp Vibrio pathogens</i>	284
G. Seghal Kiran*, Saba Rathnam, Sethu Priyadharsini and Joseph Selvin University of Pondicherry, India	
<i>A Meta-Analysis of Essential Amino Acid Requirements of Fish</i>	303
Guillaume Salze*, Quinton Margaret, and Bureau Dominique P. University of Auburn, USA	
<i>Diversification of Fads2 finfish species: Implications in aquaculture</i>	338
Naoki Kabeya*, Goro Yoshizaki, Douglas R. Tocher, Óscar Monroig University of Tokyo, Japón	
<i>Non-coding RNAs: uncovering their potential relevance in fish nutrition</i>	363
Ignacio Fernández Monzón*, Carlos Alfonso Alvarez-González, Dariel Tovar-Ramírez and Mario Galaviz Universidad de Algarve Portugal	
<i>Enfoques transcriptómicos en el jurel Seriola rivoliana</i>	390
Dariel Tovar Ramírez*, Teles, A., Salas-Leiva, J.S., Hernández-Contreras, A., Asencio-Alcudia, G.G., Le Du, J., Burgoin-Cota, M., Alvarez-González, C.A., Llera-Herrera, R., Gisbert, E., Fernández, I., Pérez-Urbiola, J.C., Ibarra-Castro, L., Mazón-Suástegui, J.M., Núñez-Vázquez, E.J., Guzmán-Villanueva, L.T., Reyes-Becerril, M. Centro de Investigaciones Biológicas del Noroeste Unidad La Paz, México	
<i>Change in protein digestion capacity during juvenile fish ontogeny: Approach on spotted rose snapper (Lutjanus guttatus)</i>	408
Emyr Peña*, Crisantema Hernández, Carlos Alfonso Álvarez-González, Leonardo Ibarra-Castro, Patricia Domínguez-Jiménez Universidad Juárez Autónoma de Tabasco, México	
<i>Synthetic growth hormone secretagogue GHRP-6 exhibits enhanced growth activity and immune system stimulation in teleost fish and shrimp</i>	432
Rebeca Martínez Rodríguez*, Yamila Carpio, Liz Hernandez, Antonio Morales, Fidel Herrera, Reynold Morales y Mario Pablo Estrada. Centro de Ingeniería Genética y Biotecnología, Cuba	
<i>Protein and amino acid nutrition of marine fish species</i>	439
Helena Peres* and Aires Oliva-Teles Universidade do Porto	
<i>Role of Marine Metabolites in Shrimp Growth, Production and Disease Prevention</i>	498
Anuj Nishanth Lipton*, Joseph Selvin, Seghal Kiran and Aaron Premnath Lipton University of Pondicherry, India	
<i>Evaluación de la Atractabilidad, Palatabilidad y Consumo de Ingredientes en Alimentos Balanceados para el Camarón Blanco del Pacífico Litopenaeus vannamei</i>	523
David A. Villareal Cavazos*, Juan Pablo Hinrichsen, Julián Gamboa-Delgado, Martha Nieto-López, Mireya Tapia-Salazar, Maribel Maldonado-Muñiz, Denis Ricque-Marie y Lucía Elizabeth Cruz-Suárez Universidad Autónoma de Nuevo León, México	
<i>Uso Profiláctico de Aditivos Inmunoestimulantes en el Cultivo del Camarón Blanco, Litopenaeus vannamei</i>	541
Ángel I. Campa-Córdova*, Jesús A. Valenzuela-Chávez, Jocelyne García-Armenta, Diana Medina, Alan B. Licon-Jain, Carlos E. Angulo-Valadez, Gabriel Aguirre-Guzmán, Claudio H. Mejía-Ruíz	

<i>Enteritis inducida por la harina de soya en las dietas de peces marinos: efectos sobre la integridad del intestino distal y la respuesta inmune en <i>Totoaba macdonaldi</i></i>	560
José Pablo Fuentes-Quesada, Artur N. Rombenso, Yanet Guerrero-Rentería, María Teresa Viana, Juan Pablo Lazo, José A. Mata-Sotres*	
CATEDRAS-Universidad Autónoma de Baja California, México	
<i>Efecto de la Dieta en el Microbioma Intestinal de Organismos Acuáticos</i>	570
Bruno Gómez-Gil*, Karen Enciso-Ibarra, Elizabeth Cruz-Suárez, Crisantema Hernández-González, Elizabeth Osuna-García, Martha Nieto-López, Clara Montero-Lizárraga.	
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Insights into the Potential of Pre-Processing of Ingredients to Improve their Economical Value to Aquaculture Species

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Abstract

Processed animal protein ingredients are valuable ingredients for aquaculture feed formulations. However, the variability of the chemical composition of different batches of these ingredients and the relatively low digestibility of some of the nutrients (i.e. amino acids, phosphorus) occasionally represent significant limitations for these ingredients at high levels in the diet of some species.

Research efforts carried out at the University of Guelph explored the potential of simple and potentially cost-effective processing techniques to improve the digestibility and nutritive value of these ingredients. Processing aimed at improving digestibility of phosphorus showed that incubation with different organic acids and a chelating agent and fine grinding significantly improved *in vitro* bio-availability of bone phosphorus of high ash poultry by-products meal. However, this processing offered no advantage in terms of *in vivo* digestibility of phosphorus to rainbow trout, a species with an acid stomach. The technique may be useful for species lacking an acid stomach (e.g. carps, shrimp) but this hypothesis has not been verified. In another series of research efforts, incubation of feather meals with protease and a reducing agent, aiming to disrupt residual disulphide bonds and cross-linkage of keratin, significantly improved *in vivo* digestibility of protein and amino acids and bio-availability of arginine of this ingredient to rainbow trout.

The results illustrate the potential of simple processing techniques, based on sound chemical principles, to improve the bio-availability of nutrients of processed animal protein ingredients. However, careful animal assays need to be carried out to confirm the usefulness of these techniques in different species.

Keywords: Processing, ingredient, protein, phosphorus, fish

1. Introduction

The aquaculture feed industry is one of the fastest growing animal feed industries in the world. It currently represents about 40 million metric tons (MMT) market which is expected to increase significantly over the next decades. Aquaculture feeds are the most expensive animal feeds available on the market and generally require the use of relatively high quality ingredients. There has been numerous calls to make better use of common by-products or under-valorized waste streams from the agriculture, food processing and industrial sectors in aquaculture feeds to improve sustainability of the aquaculture industry. Simple processing techniques could be used to improve the nutritive value and address the various limitations of by-products from various industries and ensuring that these adequately fit the needs of the different sectors of the aquaculture feed industry (e.g. high value feeds for highly carnivorous fish species vs. lower value feeds for omnivorous fish).

The rendering industry produce high quality feed ingredients, such as meat and bone meal, poultry by-products meal, feather meal and blood meal, from a variety of by-products from the animal agriculture and meat processing sectors. These ingredients are finding wide use in aquaculture feed formulations worldwide. The variability of the chemical composition of different batches of these ingredients and the relatively low digestibility of some of the nutrients (i.e. amino acids, phosphorus) occasionally represent significant limitations for these ingredients at high levels in the diet of some species.

The manuscript presents a brief overview of some of the research efforts that examined the potential of simple processing techniques, based on sound chemical principles, to improve the bio-availability of nutrients and nutritional value of rendered animal protein ingredients.

2. Improving Digestibility of Phosphorus in High Ash Animal Protein Ingredients

Research efforts carried out at the University of Guelph explored the potential of simple and potentially cost-effective processing techniques to improve the digestibility of phosphorus (P) in high ash animal protein ingredients.

2.1 Digestibility of Phosphorus

Phosphorus (P) has an essential role in cellular functions. P, in the form of phosphate, is involved in numerous structural and metabolic roles, such as bone mineralization, components of phospholipids (DNA, RNA and nucleotides), and enzyme cofactor. However, phosphates are a problem when present in excess in the aquatic environment since they are a limiting factor for algal growth in freshwater, and can consequently aggravate the eutrophication process. As a result, the management of P waste outputs by freshwater fish culture operations has been a major area of focus (Bureau & Hua, 2010). Ensuring adequate supply of digestible P without great excess is an issue of increasing significance to aquaculture feed manufacturers.

Formulating feeds to a precise digestible P content can be a difficult task. The P concentration in ingredients commonly used in aquaculture diets is highly variable. In addition, P is a component of a very large variety of chemical compounds and is therefore found in many different chemical forms. These chemical forms of P have different digestion dynamics which are affected by different factors, and this results in variability in the digestibility of P contributed by feed ingredients to the diet (Hua & Bureau, 2006; Hua *et al.* 2010).

Hua Bureau (2006; 2010) developed models that estimated the digestibility of P compounds for three commercially important groups of fish species: salmonids (Hua & Bureau, 2006), tilapia (cichlids), and carp (cyprinids) (Hua & Bureau, 2010). These authors classified the different chemical forms of P into six broad categories: bone-P (hydroxyapatite), organic-P, phytate-P, phytase effect on phytate-P, and monobasic and dibasic inorganic P. Significant differences in the digestibility of the different P compounds for each fish species were observed in these studies. This modeling exercise showed that cyprinids had a very limited

ability to digest low solubility P compounds. The digestibility of bone-P was estimated as nil to cyprinid fish species where it is about 60% to salmonid fish species and about 70% in tilapia (Hua & Bureau, 2010). Solubilization of compounds containing inorganic P in the stomach is the important factor for P digestibility (Nakamura, 1985). Bone P (hydroxyapatite) is the most stable and least soluble calcium phosphate and its digestibility is thus likely limited by gastric acid output.

Sustained interaction with feed formulators in Southeast Asia suggests that high levels of inorganic P supplements (approximately 20 kg/metric ton) are required to maximize weight gain and meet the digestible P requirement of cyprinids species. This need to supplement low value aquaculture feeds with high levels of P supplements is generally costly. There is great economical interest in increasing the digestibility of P already present in the feeds, notably the bone P contributed by animal protein ingredients.

2.2 Potential of Organic Acids

Organic acids have been used to improve P (and other mineral) utilization in fish. Dietary supplementation of citric acid, sodium citrate, and EDTA was able to improve P digestibility of fish meal to rainbow trout (Sugiura *et al.* 1998). Formic acid supplementation significantly improved P digestibility and retention in trout (Vielma & Lall, 1997). The positive effect of organic acid is probably due to the solubilisation of bone minerals, as well as a chelating effect that reduces the precipitation of Ca and P at the intestinal brush border (Sugiura *et al.*, 1998; Sarker *et al.* 2005). However, high level of free form organic acids in feeds can result in reduced palatability and feed intake which could ultimately negatively affect the growth performance of the animal. An alternative approach may be to pre-treat high bone P ingredients with organic acids in order to improve their available mineral content prior to use in feeds. Pre-treatments could potentially be done with pure organic acids or through fermentation with micro-organisms. Soil fungus and bacteria, in association with the rhizosphere have developed mechanisms to solubilize inorganic phosphorus (Pi) and make it available to the plant. This unique ability by micro-organisms has been used in the agriculture industry to enhance soil Pi uptake by plants. The mechanism used by soil micro-organisms

to solubilize Pi involves the production of organic acids that break down hydroxyapatite minerals into mono- or dibasic phosphates. These more soluble forms of Pi are therefore more available for uptake by plants. A variety of soil micro-organisms able to solubilize inorganic P have been described and a number of inoculants are available commercially.

Fermentation of feed ingredients with microorganisms may be an option but this process is costly, complex and risky. Short incubation with organic acids may be the only viable option. One of the issues with the solubilisation of bone P with organic acids is the release of large amount of calcium ions concurrent to the release of phosphate ions. These calcium and phosphate ions have a natural tendency to precipitate together when the pH is neutral. Chelating calcium ions may prevent this precipitation and improve the effectiveness of the organic acid treatment.

2.3 Validation of an *in vitro* P Bio-Availability Assay

Examining the value of different processing and incubation techniques requires a reliable *in vitro* assay to predict the *in vivo* digestibility of P of processed ingredients. The *in vitro* phosphorous bio-availability assay (PBA assay) was identified as a potentially useful tool to rapidly screen and/or predict the *in vivo* digestibility of P of feed ingredients developed during the research efforts carried out at the University of Guelph.

Extensive testing indicated that the PBA assay was not suitable for estimating the bio-availability of P in low processed animal protein ingredients, such as blood meal and feather meal (results not shown). For high ash, high bone P, animal protein ingredients, preliminary results indicated that grinding had a very significant ($p < 0.05$) positive effect on the PBA values (Table 1). This suggested that "particle size" has a significant effect on the estimate of P bioavailability obtained with the PBA assay. To confirm this, a comprehensive study was carried out to characterize the effect of particle size on estimates of P bioavailability obtained with the PBA assay.

A very strong effect of grinding (particle size) within same batch of ingredient (Table 2). However, across batches of similar ingredients, the effect of particle size is not consistent. In the case of poultry by-products meals (meals with P content < 3%), there does not appear to be any significant relationship between particle size and *in vitro* P bioavailability (Figure 1). Conversely, for poultry bone meal (meals with P content > 6%), a very strong negative association between particle size and *in vitro* P bioavailability is noted (Figure 2).

Table 1. Results of preliminary study on the effect of grinding on the bioavailability P content (estimated with the *in vitro* PBA assay) of animal protein ingredients before or after grinding.

Ingredients	Original Particle Size	After Grinding
	%	
Poultry bone meal (Batch 1)	4.32±0.02 ^B	6.53±0.07 ^A
Poultry bone meal (Batch 2)	4.39±0.02 ^B	5.59±0.27 ^A
Low Ash Poultry By-Products Meal	1.37±0.03 ^A	1.32±0.02 ^A
Regular Poultry By-Products Meal	1.7±0.00 ^A	1.69±0.00 ^A

Values in the same row sharing the same subscript are not significantly different

This particle size effect is not expected to be physiologically meaningful since grinding has been shown to have no effect on apparent digestibility of P on *in vivo* digestibility trials with salmonids (Lall, 1991). However, it suggests that "methodological artifact" appear to severely limit the value of the PBA assay to predict the bio-availability of P *in vivo*. Taken alone, *in vitro* P bioavailability estimates may be misleading. However, it was concluded that the PBA assay could probably be used as a screening tool to determine the effectiveness of "treatment" within batches of the same ingredient. However, more efforts should be invested in *in vivo* assessment of P bioavailability, through digestibility or bio-availability (growth trials) which should produce more reliable and meaningful results than *in vitro* assays.

Table 2. Bio-availability of P (estimated with the *in vitro* PBA assay) of different animal by-products prior and after grinding

Ingredient	Size	Total P %	Bioavailable P %	P Bioavailability %	Mean particle size mm ²
Fish meal, herring W	Original	2.75±0.12	1.93±0.12	70 ^A	0.06
	Ground	2.78±0.06	2.02±0.03	73 ^A	0.06
Fish meal, herring Y	Original	2.83±0.15	1.70±0.06	60 ^B	0.22
	Ground	2.84±0.49	1.96±0.05	69 ^A	0.18
Fish meal, herring Z	Original	2.76±0.18	1.70±0.04	65 ^B	0.26
	Ground	2.72±0.01	1.91±0.02	70 ^A	0.09
Meat bone meal Q	Original	3.75±0.48	2.27±0.23	61 ^B	0.23
	Ground	3.68±0.06	2.70±0.06	73 ^A	0.19
Poultry bone meal L	Original	7.04±0.30	5.12±0.13	73 ^B	0.14
	Ground	7.00±0.11	5.42±0.09	77 ^A	0.10
Poultry bone meal M	Original	6.56±0.37	4.49±0.29	68 ^B	0.20
	Ground	6.85±0.16	5.72±0.12	83 ^A	0.08
Poultry bone meal N	Original	6.91±0.36	4.66±0.19	67 ^B	0.18
	Ground	7.11±0.02	5.95±0.10	84 ^A	0.07
Poultry by-products meal A	Original	2.71±0.02	1.72±0.03	63 ^B	0.14
	Ground	2.64±0.03	1.89±0.10	71 ^A	0.12
Poultry by-products meal B	Original	2.56±0.49	1.58±0.07	62 ^B	0.19
	Ground	2.53±0.01	1.92±0.06	76 ^A	0.13
Poultry by-products meal C	Original	2.66±0.27	1.74±0.11	65 ^B	0.17
	Ground	2.67±0.08	2.00±0.05	75 ^A	0.16
Poultry by-products meal D	Original	2.88±0.10	1.87±0.04	65 ^B	0.14
	Ground	2.70±0.07	1.96±0.02	73 ^A	0.10
Poultry by-products meal E	Original	2.97±0.13	1.58±0.10	53 ^B	0.12
	Ground	2.65±0.11	2.01±0.12	76 ^A	0.07

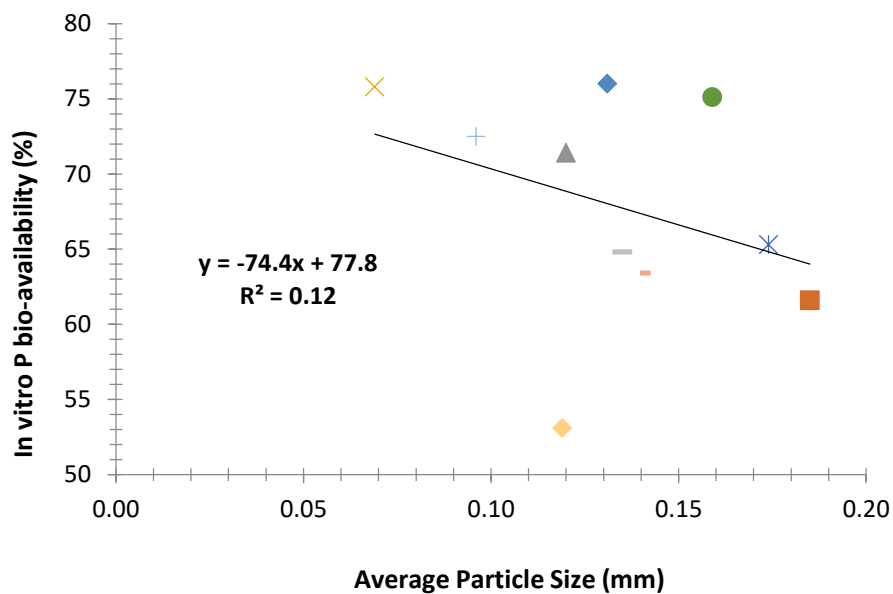


Figure 1. *In vitro* bio-availability of P of poultry by-products meals (Total P = 2.5 to 3.0%) with different average particle sizes

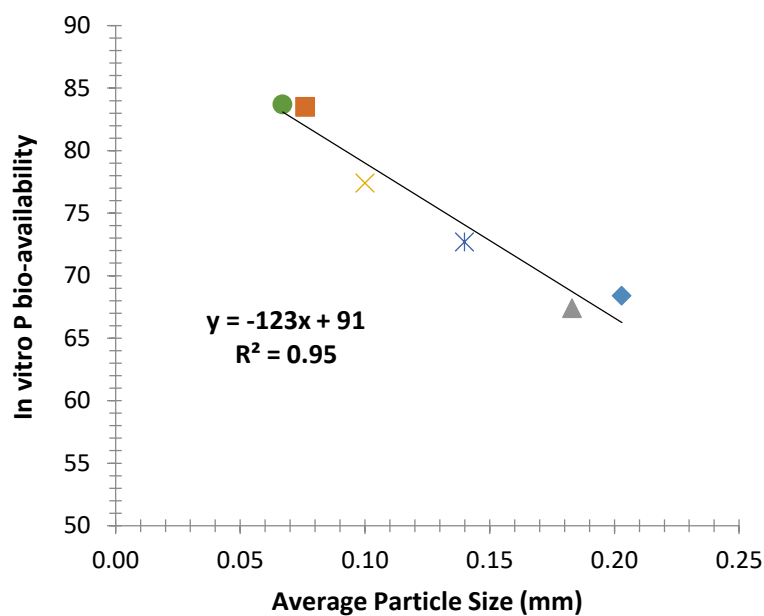


Figure 2. *In vitro* bio-availability of P of poultry bone meals (Total P = 6.6 to 7.1%) with different average particle sizes

2.4 Laboratory Bench Scale Experiments to Examine Effects of Different Treatments on *in Vitro* Digestibility of P in Poultry By-Products Meal

A series of lab bench experiments, for which the general approach is described in Figure 3, were carried out to explore the effects of different factors and reagents on the bio-availability of P in poultry by-products meal (PBM). A simple water solubilisation technique to determine the amount of soluble P that was freed up by the different treatments since we believe that this technique may more realistically estimate the digestible (available) P content of the PBM and meat and bone meals for a gastric fish species (carps) than the PBA assay described above.

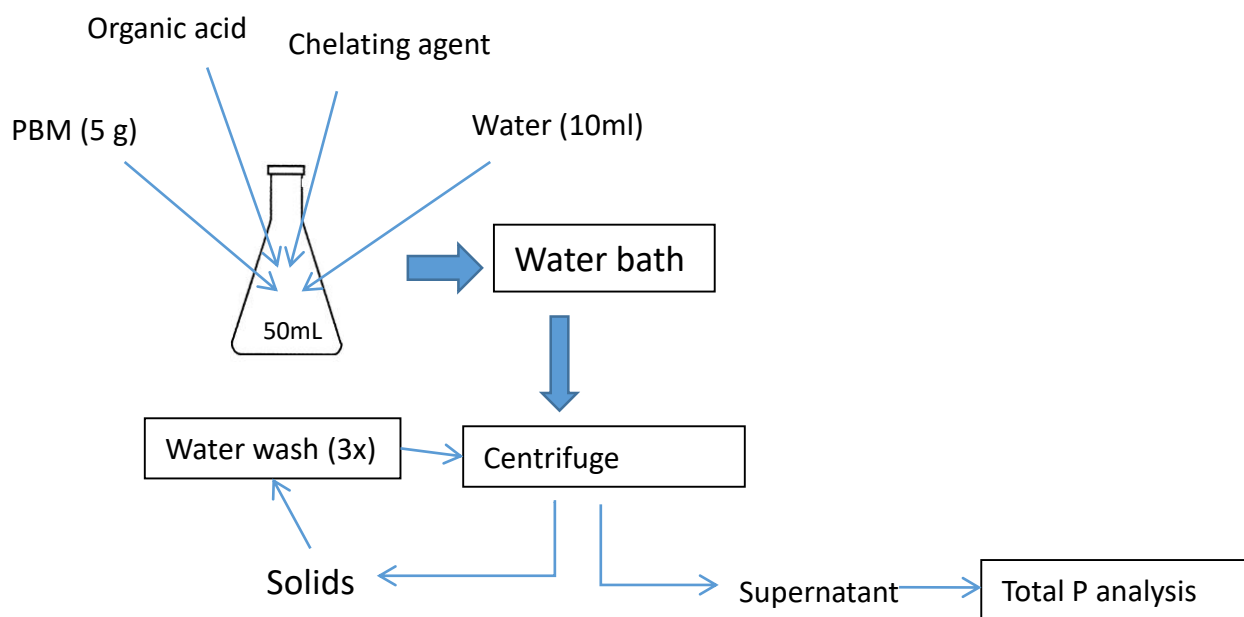


Figure 3. General approach used in the incubation trials .

This series of lab bench experiments used the same test ingredient, a relatively high ash PBM with approximately 2.6% phosphorus (as is basis) derived from a single production lot was obtained from a local rendered plant (Rothsay, Moorefield, ON, Canada). A very large proportion (> 80%) of the P content of this PBM is as estimated to be bone-P (hydroxyapatite).

A first series of experiments examined the effect of different concentration of citric acids and of a calcium chelator (EDTA). The effect of incubation period (time) and temperature were examined. The results suggest that the use of EDTA in combination with citric acid appeared to be highly effective at solubilising P. A simple graphical analysis suggests that EDTA and citric acid do not appear to act in synergy but rather have additive effects (Figure 4). The use of combinations of citric acid (0 to 10% expressed as % of total PBM weight, as is) and EDTA (0 to 9%) resulted in an increase in the soluble P level of the PBM from 0.25% to about 1.1%. These results suggested that citric acid and EDTA combinations could potentially improve the availability of P in PBM to agastric fish species from less than 10% to more than 40%.

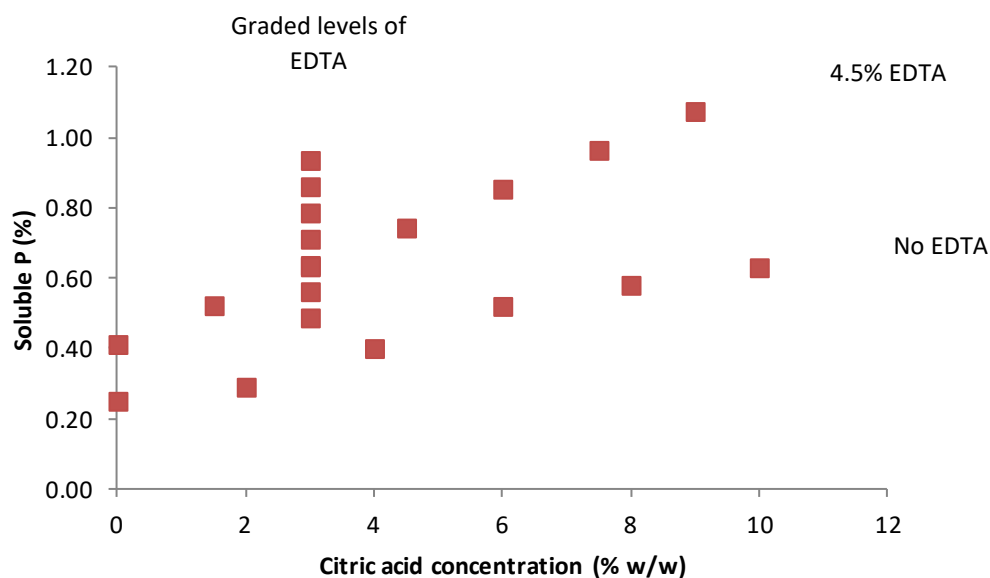


Figure 4. Effects of citric acid concentration (% w/w) on the amount of phosphorus solubilized from poultry by-products meal in the presence or absence of EDTA.

Another series of experiments compared different organic acids (or source thereof) under standardized conditions (3h at 50°C, 3.8% EDTA). These experiments suggest that citric, tartaric, oxalic formic acids were some the most effective organic acids for solubilizing bone P (Figures 5 and 6). Lactic, malic and acetic acid had moderate effects while benzoic and

ascorbic acids did not prove effective. Corn steep liquor, a source of lactic acid, did not prove effective at solubilising P in the PBM.

Of all the compounds tested, citric acid and EDTA appeared to have the greatest potential due to their reasonable cost since they are substances that have GRAS (Generally Recognized As Safe) classification by the US Food and Drug Administration (FDA).

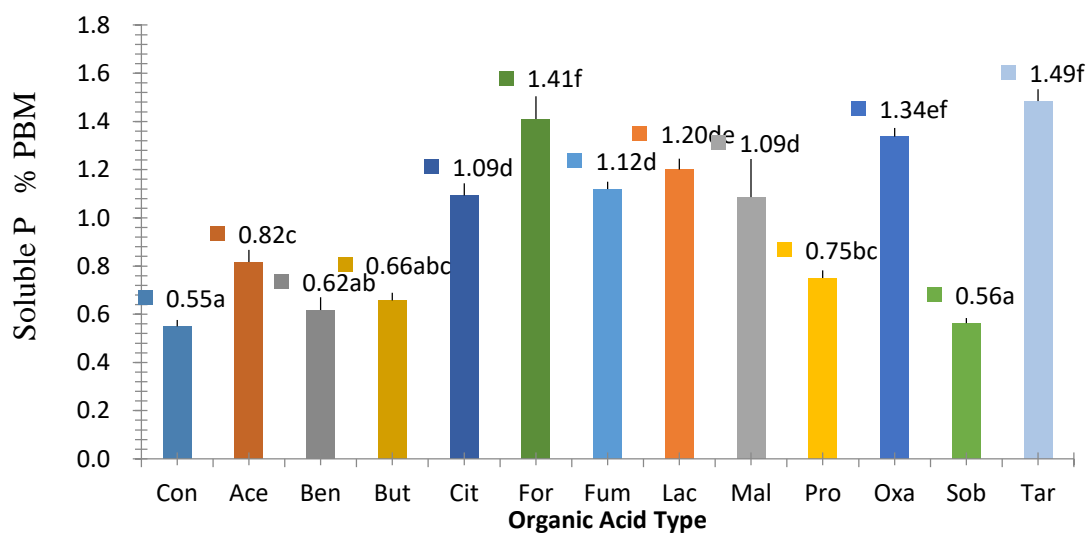


Figure 5. Soluble P obtained after incubation with 10 g 100 g⁻¹ of acetic acid (Ace), benzoic acid (Ben), butyric acid (But), citric acid (Cit), formic acid (For), fumaric acid (Fum), lactic acid (Lac), malic acid (Mal), oxalic acid (Oxa), propionic acid (Pro), sorbic acid (Sob), and tartaric acid (Tar) respectively in condition of 3.8 g 100g⁻¹ of EDTA and 65g 100g⁻¹ of system moisture

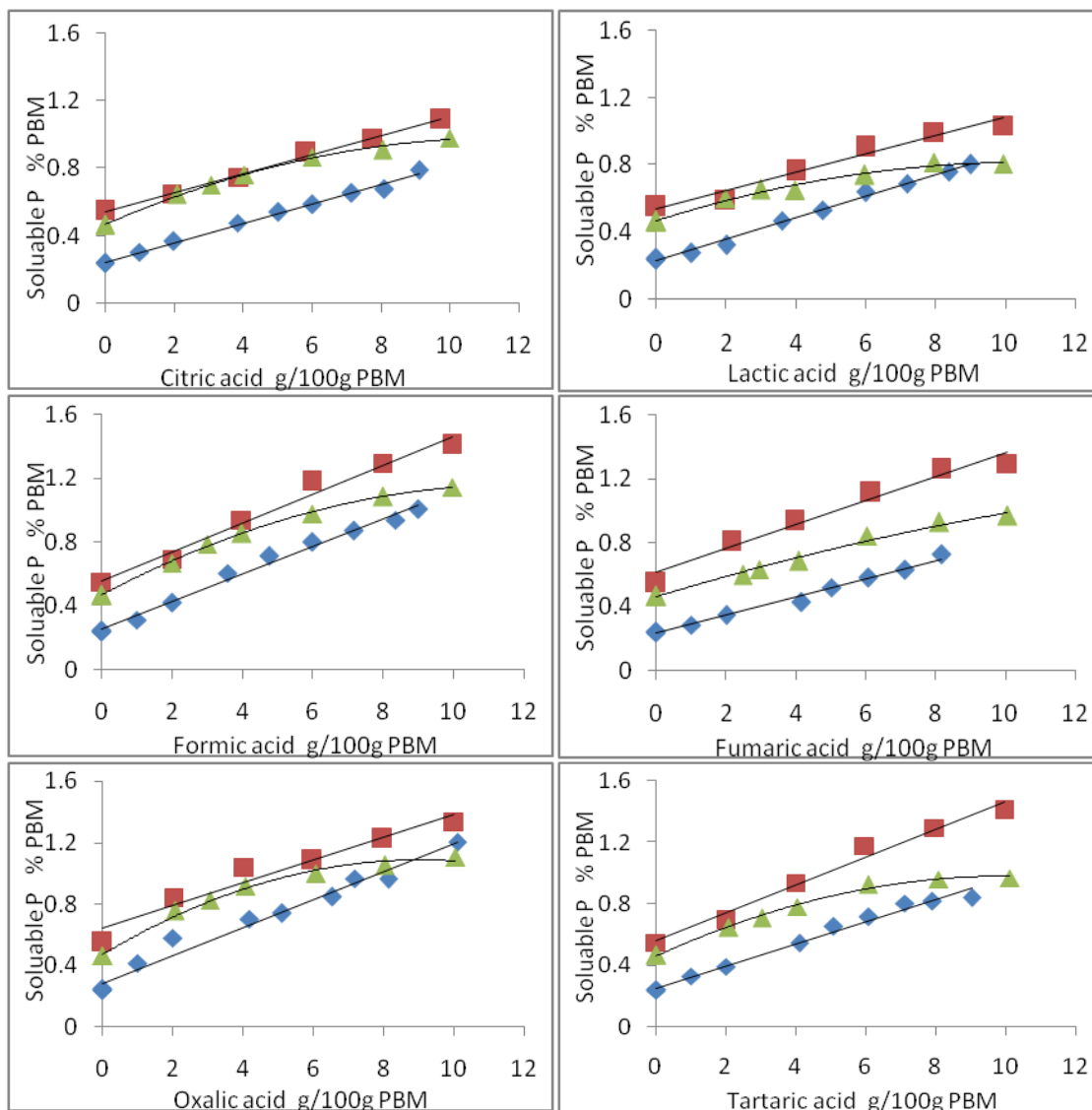


Figure 6. Effect of low molecular weight organic acids and moisture level on kinetic of phosphorus solubility from PBM.

The solubilisation of P by citric acid and EDTA treatment appeared to be very rapid (less than 1 h) (Figure 7). Increasing temperature (20-70°C) only had a very small effect on the solubilisation of P, regardless of the type of organic acid used (Figure 7) and long incubation periods (up to 200 h incubation time) did not yield any improvement in P solubility (results not shown). The incubation of PBM with a broad action protease in combination with citric acid and EDTA had no effect of the release of soluble P (results not shown).

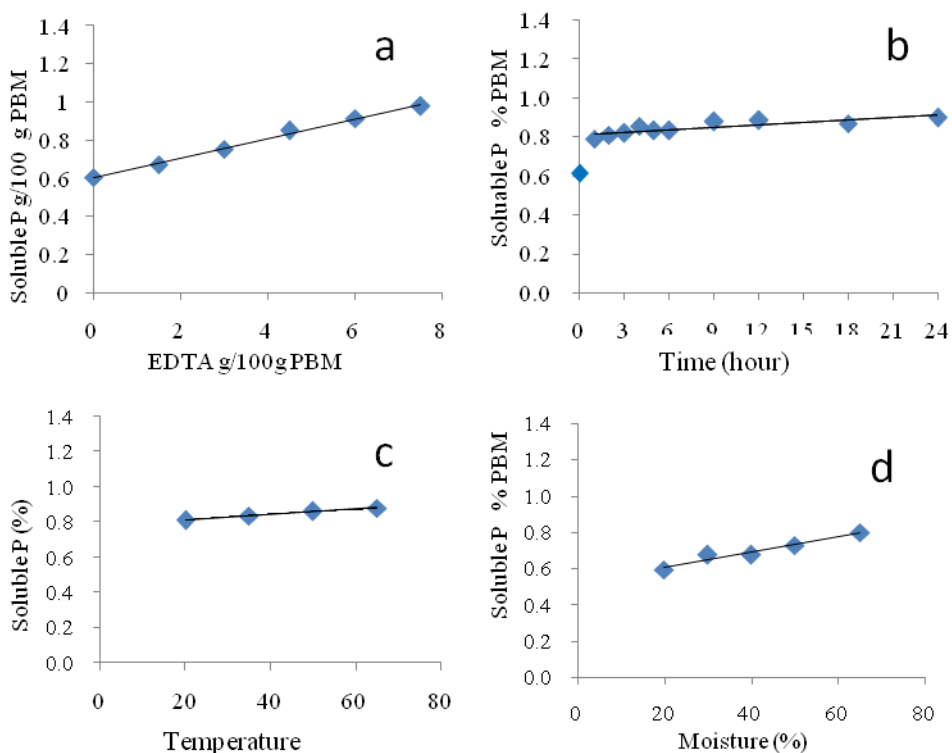


Figure 7. Effect of time, temperature, moisture and EDTA on bone P release from PBM

2.5 *In vivo* Assessment of the Digestibility of Incubated PBM and its Effects of Growth Performance

A pilot-scale trial to produce high bio-available phosphorus poultry by-products meals was conducted on the basis of the work described above. This involved producing several kg of processed PBMs with two different organic acids (formic acid and citric acid) and these two PBMs were used in a digestibility trial and a growth trial with rainbow trout carried out at the University of Guelph Fish Nutrition Research Laboratory.

2.5.1 Ingredients and Experimental Diets

The same batch of PBM described above was pre-treated by diluting 10 g citric acid or formic acid with 670 mL distilled water and adding this mixture to 1 kg PBM. After mixing thoroughly, the treated PBM was incubated at 50 °C for 3 h and then air dried. A PBM was also incubated with distilled water alone in order to have a “sham” treated PBM.

Bureau, D. et al., 2017. Insights into the Potential of Pre-Processing of Ingredients to Improve their Economical Value to Aquaculture Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. . (Eds), Investigación y Desarrollo en Nutrición Acuícola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 1-31. ISBN 978-607-27-0822-8.

A reference diet (Table 3) was prepared and combined with each test ingredient (PBM pre-treated with either water (control), citric acid or formic acid) at a 70:30 ratio (as is basis) to produce three test diets. Yttrium oxide at an inclusion level of 100 ppm was added to the reference diet to serve as a digestibility indicator. The diets were mixed using a Hobart mixer and pelleted using a laboratory steam pellet mill. The feed pellets were subsequently dried using forced air at room temperature for 24 h. The diets were kept at 4 °C until used. The proximate composition of the PBM and the reference and test diets are shown in Table 4.

Table 3. Ingredient composition of the reference diet.

Ingredients	%
Fish meal, herring, 70% CP	18.5
Soybean meal, dehulled	6.0
Blood meal, porcine, spray-dried	6.0
Corn gluten meal, 60% CP	12.0
Feather meal	5.0
Wheat middlings	12.9
Soy protein concentrate (HP300)	12.0
Vitamin premix , Martin Mills	1.0
Mineral premix Martin Mills	0.5
Wheat gluten	8.0
Fish oil	14.0
Vegetable oil	4.0
Yttrium oxide	100ppm
Total	100

Table 4. Proximate composition of the PBM, reference diet and test diets.

PBM or Diet	DM (%)	Composition (dry matter basis)					
		CP ^a (%)	Lipid (%)	Ash (%)	GE (kJ/g)	P (%)	Y ^b (µg/g)
PBM	97.4	72.0	11.3	13.9	21.8	2.46	-
Reference	94.9	53.0	20.7	5.9	24.5	0.87	88
Control	95.0	55.8	18.1	8.7	23.7	1.37	59
Citric acid	95.0	58.5	17.7	8.6	23.6	1.26	58
Formic acid	95.0	58.3	17.8	8.6	23.7	1.37	60

^aCP crude protein (N X 6.25)

^bYttrium

2.5.2 Fish and Experimental Conditions

Two trials were carried out with rainbow trout (*Oncorhynchus mykiss*) obtained from the Alma Aquaculture Research Station (Elora, Ontario). Fish were maintained in a flow-through system consisting of 60 L fiberglass tanks, individually aerated and supplied with well water at a rate of approximately 3 L/min and equipped with fecal settling columns (Guelph System) as described by Cho *et al.* (1982). Water temperature was maintained at 11.8 ± 0.5 °C and 12.6 ± 0.4 °C for the digestibility and growth trials, respectively. Photoperiod was maintained at 12 h light: 12h dark in a windowless laboratory. The animals were kept in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1984). Fish in both trials were hand-fed to satiety three times daily on weekdays and once daily on weekends.

2.5.3 Digestibility trial

Groups of 15 fish with an initial average weight of 21 g/fish were randomly distributed into 24 tanks. The four experimental diets (reference, control, citric acid, formic acid) were

randomly allocated to two collection units each. The fish were acclimated to the experimental system and dietary regime for four days prior to collection. A total of four fecal samples per diet were collected. Two fecal samples per diet were collected during the first collection period (10 days). The experimental diets were then randomly re-allocated to new collection units for the second period and two additional fecal samples per diet were collected in the following 10-day period. One hour after the last daily meal, the drainpipe and the settling column were brushed out to remove feed residues and feces from the system. One-third of the water in the tanks was drained to ensure that the cleaning procedure was complete. At 08:30 h the following day, the settled feces and surrounding water were gently withdrawn from the base of the settling column into a large centrifuge bottle. These feces were free of uneaten feed particles and considered to be a representative sample of the feces produced throughout the 24 h period. The feces were centrifuged at $4000\times g$ for 10 min and the supernatant discarded. The feces were then freeze-dried, ground and stored at -20°C until analysis.

2.5.4 Growth trial

Groups of 15 fish with an initial average weight of 35.9 g/fish were randomly distributed into nine tanks, with 3 replicate tanks per diet (water, citric acid and formic acid treated PBM). Tank was considered the experimental unit. Fish were acclimated to the experimental conditions for one week prior to the start of the experiment. Throughout the duration of the experiment (58 days), feed intake was recorded weekly and fish were weighed every 28 days. At the beginning of the experiment, a pooled sample of 12 fish was taken for determination of initial carcass composition. At the end of the experiment, five fish per tank were sampled for carcass composition analysis and an additional 10 fish per tank were weighed and dissected in order to obtain the hepatosomatic index (HSI) and viscerosomatic index (VSI).

All results were assessed for normality by the Shapiro-Wilk test, homoscedasticity by SNHT and expressed as mean values. When the data did not show normality, transformation using Box-Cox was performed prior to analysis. The dependent variables were analyzed by one-

way analysis of variance (ANOVA) followed by the Tukey's HSD test using the XLSTAT® software Version 2014.5.01. For all analyses, the level of significance adopted was $P \leq 0.05$.

2.5.5 Results

The results from the digestibility trial indicated no significant differences ($P > 0.05$) in the ADCs of DM, CP, lipid, ash, GE or P among the pre-treated PBMs (Table 5). Pre-treatment of PBM with citric acid or formic acid did not significantly improve the digestibility of this ingredient in rainbow trout.

In the growth trial, inclusion of pre-treated PBM did not significantly affect growth, feed intake and feed efficiency of rainbow trout. Excellent growth rates (TGC) were achieved with all experimental diets (Table 6). Similarly, there were no significant effects of pre-treatment of PBM with citric or formic acid on proximate and mineral carcass composition or nutrient utilization efficiency of rainbow trout (results not shown). The pre-treatment methods employed in this study did not appear to improve the nutritive value of PBM for rainbow trout. Research needs to be carried out to determine if this treatment could improve the bio-availability of P to aquaculture species without acid stomach (carps, shrimp, etc.).

Table 5. Apparent digestibility coefficients of PBM pre-treated with water (control), citric acid or formic acid in rainbow trout.

Ingredient	Apparent digestibility coefficients ¹					
	DM (%)	CP (%)	Lipid (%)	Ash (%)	GE (%)	P (%)
Control	68 ^a	78 ^a	76 ^a	38 ^a	72 ^a	43 ^a
Citric acid	69 ^a	79 ^a	78 ^a	40 ^a	73 ^a	39 ^a
Formic acid	71 ^a	80 ^a	83 ^a	40 ^a	75 ^a	45 ^a
Significance²	NS ⁴	NS	NS	NS	NS	NS
S.E.M. ³	0.0101	0.0091	0.0178	0.0088	0.0210	0.0210

¹Mean (n=4 replicates). Means in the same column sharing a common superscript are not significantly different according to Tukey's HSD test.

²Significance of the one-way ANOVA.

³S.E.M.=standard error mean.

⁴Not statistically significant ($P \geq 0.05$).

Table 6. Weight gain, growth rate, feed intake, feed efficiency (FE) and viscerosomatic (VSI) and hepatosomatic (HSI) indices of rainbow trout (initial average weight= 35.9 g/fish) fed the experimental diets for 58 days.

Diet	Gain (g/fish)	TGC ¹	Feed intake (g/fish)	FE ² (gain/feed)	VSI (%)	HSI (%)
Control	143.0 ^a	0.311 ^a	122.6 ^a	1.16 ^a	20.4 ^a	1.36 ^a
Citric acid	140.0 ^a	0.313 ^a	125.7 ^a	1.12 ^a	19.8 ^a	1.46 ^a
Formic acid	138.3 ^a	0.304 ^a	120.7 ^a	1.13 ^a	18.8 ^a	1.39 ^a
Significance ³	NS ⁵	NS	NS	NS	NS	NS
S.E.M. ⁴	2.711	0.005	2.085	0.01	0.38	0.02

¹TGC=thermal-unit growth coefficient.

²FE=feed efficiency.

³Significance=significance of the one-way ANOVA. Means in the same column sharing a common superscript are not significantly different according to Tukey's HSD test.

⁴S.E.M.=standard error mean.

⁵NS=not statistically significant ($P \geq 0.05$).

3. Improving Digestibility of Protein and Amino Acids in Feather Meal

3.1 Overview

Hydrolyzed feather meal (FeM) is a relative economical feed ingredient with high protein content (78-87% CP). This ingredient is manufactured from feathers, a co-products of the poultry industry. In 2012 only, 60 billion chickens were raised and processed globally (FAO stats). Feathers represent from 5 to 7% percent of the chicken body weight (Williams et al. 1991). Consequently, extremely large volumes of feathers are generated annually around the globe (Poole *et al.* 2008; Huda & Yang 2009). Other keratinous material (hair, wool, horn, hoof, claws, etc.) also represent also very important protein resources globally.

Raw feathers are made of over 90% of keratin, a protein that is also the main structural component of hair, nails, wool, horns and claws. Keratin contains approximately 7% cysteine and this amino acid forms disulphide bridges with other cysteine molecules. Together with hydrogen bonds, these bonds tightly package the polypeptides in a filamentous structure to form a helical array of twisted β -sheets that gives strength to the keratin molecule (Fraser et al. 1972). The disulphide bonds also render keratin highly resistant to the action of proteases. Due to their very high keratin content, feathers are virtually indigestible in their natural state. Several studies have shown that raw feathers are indigestible by cats, dogs, chicks, owls and rats (Mangold & Dubiski 1930; Moran *et al.* 1966). The strong internal interactions make keratin extremely resistant to gastric solvents and proteolytic enzymes (Wrzesniewska-Tosik and Adamiec 2007).

Steam hydrolysis commonly be used to process raw feathers into feather meal. Exposing feathers to high moisture, pressure and heat creates conditions that promote the rupture of the disulphide bridges and of the hydrogen bonds, which dissociate the proteins, allowing to the amino acids in keratin to be accessible to protease and allow keratin to become digestible which free up amino acids and render them potentially bioavailable. Finally, the steam-treated feather biomass is then dried and grounded to produce feather meal. Feather meal is generally rich in arginine, cysteine and threonine, but deficient in methionine, lysine, histidine and tryptophan.

The chemical composition, notably the high digestible protein and amino acid contents of feather meal makes it, in theory, a highly interesting ingredient for animal feeds, notably feeds for aquaculture species which are often formulated to very high (> 30%) protein levels (Bureau *et al.* 1999). However, negative perceptions exist among aquaculture feed manufacturers about the stability of the nutritive value of this ingredient. Some feed industry stakeholders believe that the composition, the *in vitro* digestibility (pepsin digestibility assay) and growth performance achieved with this ingredient are much too variable and relatively unpredictable to be used at a significant level in commercial feed formulations.

Processing equipment and conditions used by renderers are relatively highly diverse, each renderers using slightly different mixes of raw material (feathers, hog hair, etc.), cooking, drying and grinding equipment and operating conditions (temperature, pressure, duration, etc.). Variability in the nutritional value of FeM results from the use of different raw materials and processing conditions during the production of this ingredient (Latshaw, 1990; Latshaw *et al.* 1994; Wang and Parsons, 1997; Bureau *et al.* 1999). Variations of heat processing conditions create disparities of disulphide conformers, D-amino acids, and cross-linked amino acids contents, and these affect the nutritional value of protein ingredients. Better understanding of the chemical interactions occurring during the processing of FeM would enable the development of methods to improve and estimate the nutritional value of this ingredient, which could assure the quality of FeM to feed stakeholders.

3.2. Enzymatic Treatment and Fermentation to Improve Digestibility of Feather Meals

Over the past two decades, a great deal of effort was invested in improving the nutritional value of feed ingredients through enzymatic treatment. Most of the work involving the enzymatic treatment of feathers focused on the fermentation of raw material using keratinolytic bacteria such as *Bacillus subtilis* or *B. licheniformis* isolated from keratin wastes. Microbial fermentation requires cultivation of bacteria and incubation in a controlled environment which is logistically difficult to perform on a commercial scale. Studies suggested that the bacterial degradation of keratin is accomplished through of the combined action of disulphide reductases and proteases (Yamamura *et al.* 2002; Ramnani *et al.* 2005).

The reductases break the disulphide bridges between the cysteine amino acids and allow to the proteases to cut down the peptides (Bockle and Muller 1997). Ramnani and Gupta (2007) almost completely degraded feather keratin (96%) using Savinase® (Novozymes), a serine protease from *Bacillus sp.*, and sodium sulfite as a reducing agent. It has been found that sodium sulfite can reduce the cystine-disulfide bonds abundant in keratin to produce a cysteine thiol and a Bunte salt (Elashi *et al.* 2013, Figure 8). This reduction of the cysteine-disulphide bonds destabilizes the protein and in theory render it more susceptible to proteolytic enzymes.

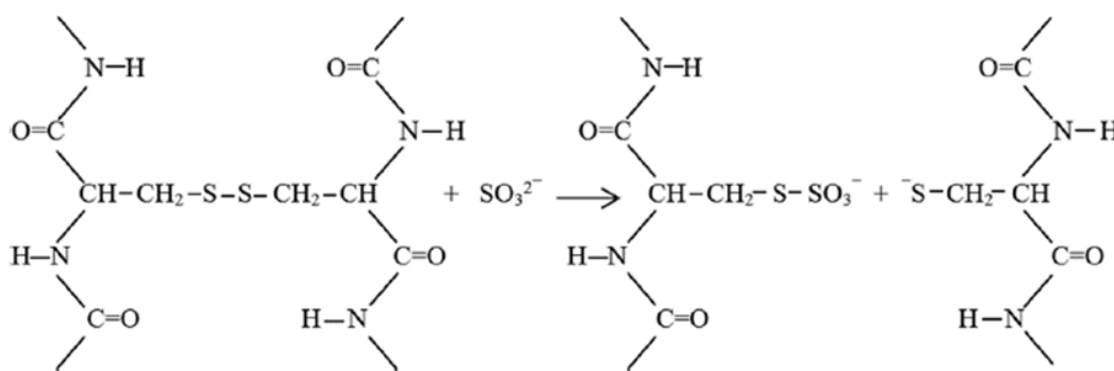


Figure 8 The addition of a sulfite to a cystine-disulfide bond to produce a cysteine thiol and a Bunte salt

Based on proprietary research and development (R&D) work around this process, Dupont Nutrition Biosciences APS (Copenhagen, Denmark) applied for US Patent Pub. No. US 2015/0197783 A1 “Method for the Degradation of Keratin and Use of the Keratin Hydrosate Produced”. The work described in the patent is almost solely laboratory bench-scale studies with keratinous material and only theoretical use of the “keratin hydrolysate” in animal feeds is described. No pilot-scale trial and animal trials appeared to have been conducted in order to assess the nutritive value of the resulting material.

The procedure also substantially raises the cost of production as it requires expensive reagents (enzymes, buffers, and reducing agent), supplementary equipment, and addition of water, demanding additional energy for drying. There was consequently a need for validation

of this technology through animal assays. There is also a need to scale up the procedure and examine feasibility, cost and actual economic value of the processed ingredients.

3.3 Pre-Processing of Steam Hydrolyzed Feather Meal

3.3.1 Introduction

The purpose of a study conducted at the University of Guelph was to examine the effect of two different proteases and a chemical agent (sodium disulfite) able to reduce the cysteine-disulphide bonds abundant on the hydrolysis on the *in vitro* hydrolysis of keratin. The goal was to try to develop an optimized protocol for the pre-treatment of feather meal prior to its use in animal feeds. An *in vitro* estimate of digestibility, the degree of protein hydrolysis (degree of hydrolysis DH) was used as a measure of effectiveness for keratinolysis after enzymatic and reducing agent treatment.

3.3.2 Material and Methods

A full factorial model lab-bench study was conducted to investigate the effect of 1) protease level, 2) sodium sulphite level, and 3) digestion buffer level on the degree of hydrolysis of feather with using two different enzymes.

This study examined the effect of two different proteases: a fungal protease (Protease A) and a bacterial protease (Protease B), sodium sulfite and water on hydrolysis of protein of feather meal samples according to a multifactorial (2x3x3x4) design with 36 treatments (Table 7). Each hydrolysis trial were conducted on a bench scale basis on sample (approximately 20 g) of a commercial feather meal (Sanimax, Montreal, Qc, Canada). The trials were performed at the temperatures and pH recommended by the enzyme manufacturers for a period of 3h. The reaction will be stopped by adding one volume of 20% trichloroacetic acid (TCA). Each treatment combination was tested in three separate incubation (triplicates). In this study, a total of 216 feather meal samples were incubated.

Enzymatic Digestion

The digestion buffer was prepared by mixing distilled water with Trizma base® (1.211 g/L) and sodium azide (0.10 g/L) thoroughly at room temperature (Coll *et al.* 2007). The pH of the digestion buffer was adjusted to meet the enzyme manufacturer's recommendations with a pH of 10.0 for Protease B and a pH of 7.5 for AG175. Each sample was incubated for a period of 3 hours in a shaking water bath at temperatures attuned to the reported optimal activity of the proteases, namely 37°C for the fungal protease (Protease A) and 55 °C for the bacterial enzyme (Protease B). The samples were centrifuged (4,000 rpm, 10 minutes) and the supernatant will be collected to determine degree of hydrolysis.

The efficiency of each combination of parameters to hydrolyze FeM was assessed as the percentage of nitrogen solubilized in trichloroacetic acid solution by the pre-treatment and expressed as degree of hydrolysis (DH).

Table 7. Experimental design of the hydrolysis trial

Independent Variables	Levels			
P= Protease	A	B		
A= Water:FeM ratio	2:1	3:1	4:1	-
B= Enzyme level (% FeM)	0	1	2	3
C= Sodium Sulfite Level (% FeM)	0	1.5	3	-

3.3.3 Results

The results from this lab bench study showed that feather meal could be very effectively hydrolyzed in vitro with a mixture of protease and sodium sulfite. The addition sodium sulfite to the incubation mixture promoted the chemical reduction of the disulphide bridges and substantially unlocked the capacity of enzymes to hydrolyse FeM. Difference was found in the effectiveness of the proteases. Protease B showed a significantly greater ($P < 0.0001$) hydrolyzing capacity in comparison to Protease A.

Enzyme level of 3% (%FeM w/w), reducing agent level of 3% (%FeM w/w), and water level of 500% (%FeM w/w) was found to be the optimal conditions to hydrolyze feather meal using both enzymes. Under optimal conditions, DH of 45% was reached with Protease B.

The efficacy of the pre-treatment to improve the nutritive value of FeM in practical animal diets needs to be systematically investigated through digestibility and bioavailability trials with livestock species such as fish, poultry and swine. If proven efficient, the pre-treatment could enable a substantial increase in the incorporation of FeM in animal feeds.

Table 8. Actual levels of independent variables along with the observed values for the response variables, degree of hydrolysis for the Protease A and B.

Run Number	Independent Variables			Degree of Hydrolysis (%)	
	X1 ¹	X2 ²	X3 ³	Protease A ⁴	Protease B ⁴
1	0	0	200	9.5 ± 0.36	9.3 ± 0.14
2	1	0	200	10.9 ± 0.32	16.1 ± 0.71
3	2	0	200	11.3 ± 0.33	17.3 ± 0.48
4	3	0	200	11.6 ± 0.10	18.5 ± 0.42
5	0	1.5	200	9.5 ± 0.19	9.7 ± 0.33
6	1	1.5	200	12.6 ± 0.05	22.4 ± 1.09
7	2	1.5	200	13.9 ± 0.41	25.7 ± 0.80
8	3	1.5	200	14.4 ± 0.77	27.1 ± 0.31
9	0	3	200	9.4 ± 0.74	9.8 ± 0.51
10	1	3	200	13.5 ± 0.70	27.4 ± 1.32
11	2	3	200	16.0 ± 0.87	31.2 ± 0.37
12	3	3	200	16.8 ± 0.84	32.9 ± 0.60
13	0	0	350	9.4 ± 0.16	9.4 ± 0.30
14	1	0	350	11.1 ± 0.29	17.0 ± 0.22
15	2	0	350	11.5 ± 0.21	18.7 ± 0.13
16	3	0	350	12.0 ± 0.27	21.2 ± 0.59
17	0	1.5	350	9.6 ± 0.27	10.2 ± 0.31
18	1	1.5	350	13.8 ± 0.04	27.4 ± 0.57
19	2	1.5	350	15.1 ± 0.64	30.7 ± 0.68
20	3	1.5	350	16.1 ± 0.08	31.8 ± 1.56
21	0	3	350	10.6 ± 0.28	10.3 ± 0.38
22	1	3	350	17.6 ± 0.76	36.2 ± 0.39
23	2	3	350	20.7 ± 1.16	40.1 ± 0.25
24	3	3	350	21.8 ± 1.16	43.5 ± 1.62
25	0	0	500	9.7 ± 0.44	10.0 ± 0.42
26	1	0	500	11.5 ± 0.36	18.6 ± 0.06
27	2	0	500	11.5 ± 0.22	20.4 ± 0.76
28	3	0	500	12.4 ± 0.25	22.0 ± 0.82
29	0	1.5	500	10.3 ± 0.35	10.2 ± 0.08
30	1	1.5	500	14.0 ± 0.27	28.8 ± 0.79
31	2	1.5	500	15.4 ± 0.17	30.5 ± 0.76
32	3	1.5	500	17.3 ± 0.55	33.2 ± 0.37
33	0	3	500	10.1 ± 0.27	10.7 ± 0.28
34	1	3	500	17.4 ± 0.65	38.3 ± 1.85
35	2	3	500	20.4 ± 0.28	39.3 ± 1.68
36	3	3	500	23.2 ± 0.22	45.1 ± 1.62

¹ X1=Enzyme level (%FeM w/w)

Bureau, D. et al., 2017. Insights into the Potential of Pre-Processing of Ingredients to Improve their Economical Value to Aquaculture Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. (Eds), Investigación y Desarrollo en Nutrición Acuicola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 1-31. ISBN 978-607-27-0822-8.

² X2=Sodium sulfite level (%FeM w/w)

³ X3=Water level (%FeM w/w)

⁴ Data are means (n=3) \pm Standard deviation.

3.4 Assessing the Bio-availability of Arginine and Digestibility of Amino Acids in two Feather Meals and in their Pre-Treated Counterparts to Rainbow Trout (*Oncorhynchus mykiss*)

The effect of this pre-treatment on the bioavailability of arginine (Arg) and on the digestibility of amino acids of two commercial steam-hydrolyzed FeMs and their pre-treated counterparts (PTFeMs). To produce pre-treated FEMs, the two commercial FeMs were incubated in 200% distilled water (%FeM w/w), with 0.05% of a protease (Protease B, %FeM w/w) and 2% sodium sulfite (%FeM w/w) at 55°C for 24 hours in a shaking incubator. The two PTFeMs were then lyophilized.

The relative bioavailability of Arg in two FeMs and their pre-treated counterparts (PTFeMs) were compared with that of crystalline L-arginine (L-Arg) in an 8-week growth trial with rainbow trout. An Arg deficient (1.2% Arg) basal diet was formulated and ten other diets were formulated to contain 1.35% or 1.5% Arg by adding increasing amounts of L-Arg, FeMs, or PTFeMs.

The results (Figure 9 and Figure 10) clearly indicated increased Arg bioavailability of the two PTFeMs compared to the two commercial FeMs. The two PTFeMs supported higher growth rates and Arg retention efficiency than their untreated counterparts. The PTFeM 1 supported an arginine retention efficiency of about 70% which is the maximum theoretical arginine retention efficiency predicted by factorial amino acid requirement models developed at the University of Guelph.

Two digestibility trials were carried out to examine the digestibility of diets formulated with the FeMs. In first trial using the diets used in the bio-availability trial, PTFeMs containing diets presented significantly ($P < 0.05$) higher apparent digestibility coefficients (ADC) of crude protein (CP) and amino acids when compared to the diets containing the original FeM counterparts. In a digestibility trial carried out with the standard 70:30 indirect digestibility protocol, the ADC of CP in FeM1, PTFeM1, FeM2, and PTFeM2 were estimated to be 85%, 95%, 82%, and 96%, respectively. The pre-treatment of these FeMs significantly improved

($P < 0.05$) their ADC of CP and amino acids (with the exception of histidine, lysine and methionine).

These findings contradict those of Serwata (2007), Laporte (2007) and Davies *et al.* (2009) who did not observe a significant difference in ADC of CP between standard steam-hydrolyzed FeMs and enzyme-treated FeMs by rainbow trout, European sea bass, gilt-head sea bream, and turbot. This appears to indicate that the presence of both a reducing agent and disulphide reductase is essential to enable the proteolytic enzymes to efficiently hydrolyze keratin into peptides and amino acids.

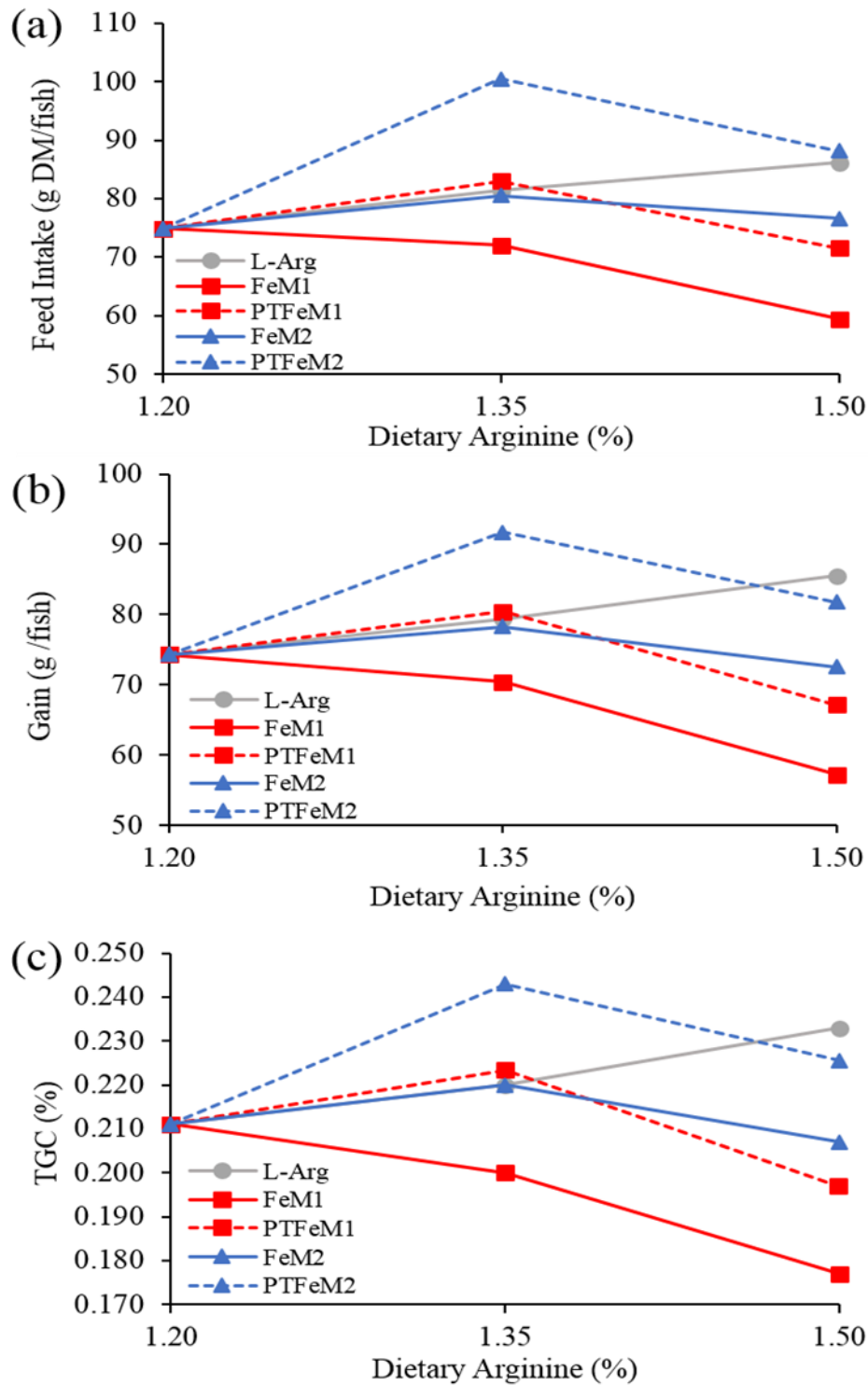


Figure 9. Feed intake (a), weight gain (b), and TGC (c) values of rainbow trout in response to being fed diets containing increasing arginine content supplied by L-arginine, FeM1, PTFeM1, FeM2 or PTFeM2. Values are mean (n = 3).

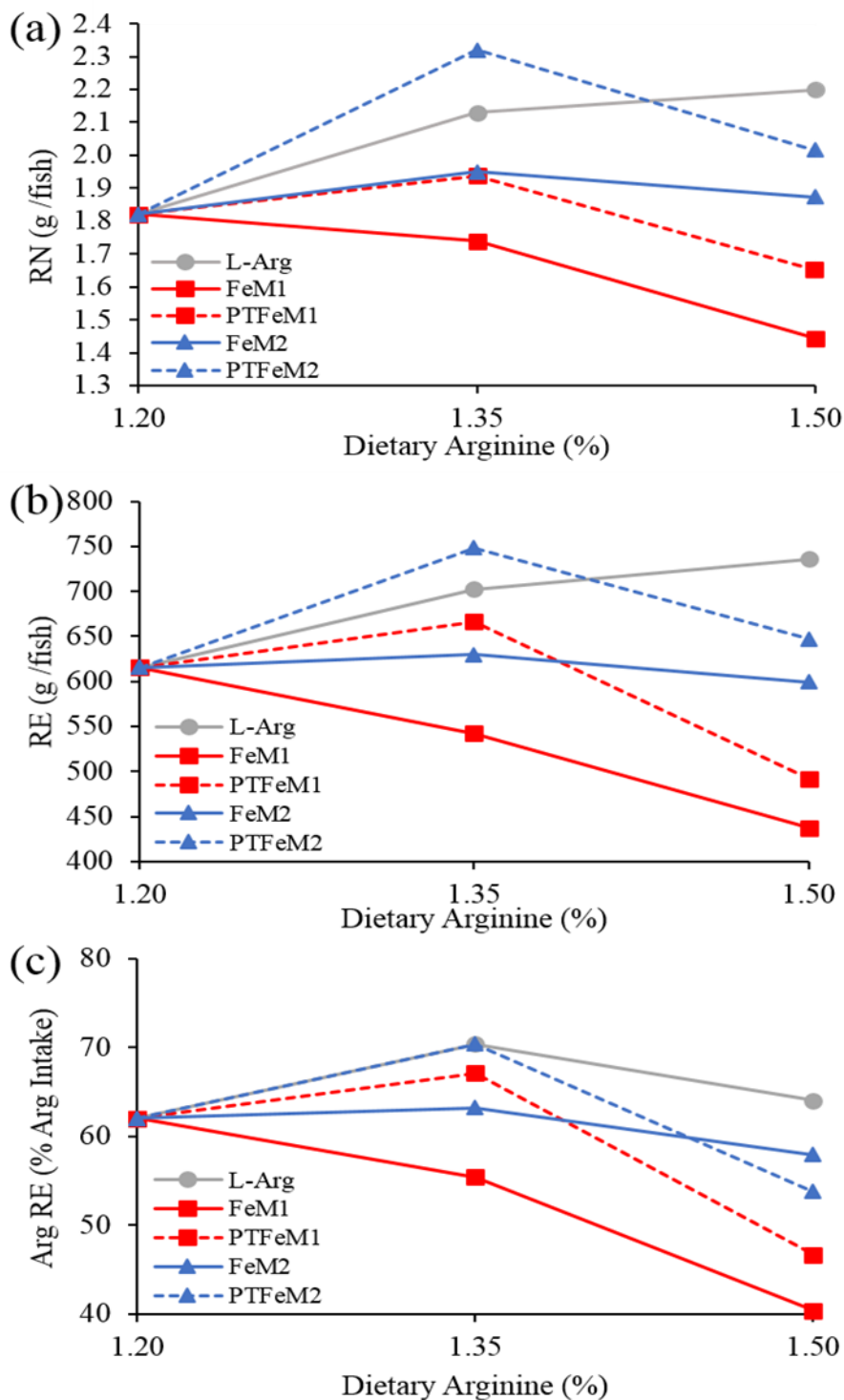


Figure 10. Retained nitrogen (a), recovered energy (b), and arginine retention efficiency (c) values of rainbow trout in response to being fed diets containing increasing arginine content supplied by L-arginine, FeM1, PTFeM1, FeM2 or PTFeM2. Values are mean (n = 3).

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Fact or Fiction: Methionine Requirement for Pacific White Shrimp

Litopenaeus vannamei

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Abstract

Considerable effort has been invested into the development and validation of alternative feed formulations for shrimp. Based on the PI's experience with the transfer of this technology to feed manufactures, the primary constraint is a poor understanding or a lack of defined studies that pinpoint the methionine or total sulfur amino acid (methionine + cysteine) requirement of shrimp. Many feed manufactures have the methionine requirement set relatively high which favors the inclusion of expensive fishmeal vs that of non-marine protein sources. Given current economic and social concerns, this is not a sustainable approach. There are several publications evaluating methionine supplements to Pacific white shrimp feeds. Yet these papers do not provide a clear definition of the requirement, which is a major constraint to feed manufactures acceptance of low fishmeal feed formulations. One theory often presented, is that crystalline amino acids are absorbed and circulate in an asynchronous patten to those from intact proteins. Based on current research we have demonstrated that the uptake of amino acids in shrimp corresponds to the digestive physiology of the animal. In that, shrimp are semi-continuous feeders that process and digest foodstuffs very quickly. From the initiation of feeding, an upswing in amino acids in the hemolymph was apparent within 10 minutes confirming very quick processing and digestion of nutrients. The clearance of amino acids was also relatively fast with amino acid levels returning to overnight fasting levels within 60 minutes of fasting. This cycle of nutrient cycling corresponds to the semi-continuous feeding habits of shrimp. Based on both absorption and clearance patterns of the amino acids, there was no indication of a synchronous absorption of supplemented amino acids; hence, crystalline amino acids should be available for metabolism. Hence, if we can produce a deficient diet, we should be able to induce a classic dose response. Across numerous growth trials, published and unpublished, the response of juvenile shrimp to a range of methionine sources have been evaluated such sources include DL-methionine, Micro-encapsulated methionine, coated methionine, synthetic peptides and various chelated forms. Yet, within the published literature there is little consistency of responses or any definitive studies. Many of these studies present contradictory or inconsistent data and hence do not provide strong evidence for a definitive requirement. Within our laboratory, across numerous independent growth trials, increasing the level of methionine as a supplement or using corn protein concentrate as an intact protein source, increasing methionine levels of the diet produced very little evidence of growth enhancement or no consistency of the response. In this authors opinion, inconsistent responses are due to other factors and the methionine requirement for this species is

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quite low and may not be a limiting amino acid in many commercial feed formulations. The alternative hypothesis, is that many of our purified forms are not available to shrimp or that test diets and systems are not appropriate. Clearly, there is a need to better understand amino acid metabolism in shrimp and define limiting amino acids as well as techniques to consistently define amino acid requirements.

Keywords: Methionine, Requirement, shrimp

Introduction

Pacific white shrimp, *Litopenaeus vannamei*, is regarded as the primary cultured shrimp species worldwide and a high value commodity with a production of around 4.5 million metric tons in 2016 (Anderson 2016). As shrimp consumption is expected to continue to increase, it is vital to develop sustainable alternative ingredients in the shrimp diets to support the expansion of the shrimp industry (Achupallas *et al.* 2016b, Achupallas *et al.* 2016a). From a nutritional standpoint, the popularity of Pacific white shrimp is due to their adaptability to a range of diets, tolerance of plant-based feeds and ability to utilize natural productivity. This species is very tolerant of diets with a range of fishmeal levels including fishmeal-free diets (Morris *et al.* 2011, Roy *et al.* 2009, Samocha *et al.* 2004).

Using proper replacement strategies (Davis 2007), several studies have demonstrated that fishmeal and marine oil levels can be reduced or eliminated from growout diets of the Pacific white shrimp. Currently, a mixture of cheaper, high-quality plant proteins (e.g. solvent-extracted soybean meal, specialized soy protein, corn protein concentrate, distillers grain solubles, pea meal) or terrestrial animal protein sources (e.g. poultry by-product meal, meat and bone meal, blood meal) can be used to successfully replace fishmeal in shrimp feeds without compromising growth or survival (Sookying *et al.* 2013). However, this can only be accomplished if shifts in nutrient requirements such as essential amino acids (particularly methionine), energy content of the diet, essential fatty acids as well as minerals such as phosphorus are accounted for (Davis 2007). Despite the preponderance of information on alternative feed formulations (see Sookying *et al.* (2013) for a review), a wide spread acceptance of low fishmeal feeds by the aquaculture industry in general, and the shrimp industry is still lacking. Although fishmeal levels in the Americas has been reduced over the years, the level of fishmeal in shrimp feeds in Asia is much higher i.e. 20% or greater of the diet (personal observation). It is well known that fishmeal supplies will not increase as most fisheries are beyond sustainable limits. A portion of this demand will be met with fisheries byproducts that are currently underutilized as well as that from processing aquaculture products. Hence, if aquaculture is to expand, the industry must move away from fishmeal as a primary protein source, particularly in production diets.

From a feed formulation standpoint, one must ask, why do we find ourselves in such a situation? First, there are very few quality studies defining the essential amino acid requirements for this species. The NRC reports a dietary methionine requirement of 0.7% and total sulfur amino acid (TSAA, methionine + cysteine) requirement of 1% for both the Kuruma and Tiger shrimp but do not report one for the Pacific white shrimp (NRC 2011). Fox *et al.* (2011) reported on a series of studies designed to evaluate digestibility, leaching and the methionine requirement of the Pacific white shrimp. Although this research confirmed digestibility of a range of methionine supplements, they were unable to demonstrate a methionine deficiency. Forster and Dominy (2006) evaluated three methionine sources supplemented to a basal diet. Albeit they reported, a deficiency in the basal diet which contained 0.45% methionine. This statement was based on a contrast in which all supplemented data was pooled. However, the authors also report ANOVA results were > 0.1 for the reported growth data. More recently, Façanha *et al.* (2016) reported on results for a methionine trial at three densities in outdoor tanks. They observed no dose response at two of the densities and a “linear” increase in weight gain in the third, with the requirement estimated at 0.72 (1.19% TSAA) to 0.81 (1.28 TSAA) using a 36% protein diet. In this author’s opinion, this is quite odd as the response should not be affected by density and there was no clear performance plateau in the shrimp for which a response was seen. In another recent publication, Lin *et al.* (2015) reported on three experiments with three different sizes of shrimp. They found almost no difference in weight gain across all experiments and treatments. Yet using regression analysis presumably of the mean values, a “dose response” to methionine was reported which estimated a methionine requirement of 0.91% diet or 2.28% of the protein in a diet with 0.5% cysteine. Once again, this is very weak data, which may not have been analyzed using the most appropriate methods (i.e. regression on treatment means), the requirement estimate is much higher than that reported for other species, and the results were not repeatable across size classes.

The definition of a requirement is further complicated by trade journal publications which have printed a range of articles which may be misinterpreted. For example, one recent article presents a survey of essential amino acids (EAA) levels in commercial feeds. The authors indicated that the average TSAA level found in feeds for Pacific white shrimp was 3.36 % of the protein. They further supported a recommendation of extremely high levels of methionine and other EAA. This report indirectly advocates for high levels of marine protein sources as that is the only way to

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reach such high level of methionine and TSA. However, these numbers clearly conflict with whole-body levels, cysteine replacement value, as well as levels published for other species. Therefore, these numbers very likely misrepresent requirements, and such reports are not doing the industry any good. With the exception of lysine (Fox *et al.* 1995), there are few classical requirement studies to refute these claims. We would suggest that a concerted effort and systematic, rational evaluation of limiting AA must be initiated in an effort to define requirement in the Pacific white shrimp through a classic approach. Such data would allow feed formulators to have the confidence to exchange protein sources based on AA profiles and cost.

With the support of a range of funding sources my laboratory has systematically developed soy-based feed formulations in shrimps (Sookying *et al.* 2013). Across numerous trial with intact protein source, we have demonstrated no response to substitutions, which have driven the methionine and total sulfur amino acid levels to very low levels, without a decrease in growth or survival. Recently our laboratory has expended considerable effort into looking at limiting amino acids in shrimp feed. The following summaries recent experimental data attempting to elucidate the methionine requirement and evaluate the efficacy of crystalline amino acids.

Methods

Diet preparation

The basal diets used in the various trials were designed to contain 34% protein and 9% lipid using primarily soybean meal and fishmeal or poultry by product meal as the primary protein sources (Table 1, 2, 3, and 4). With the exception of methionine, the basal and test diets were formulated to meet the nutritional requirements of the Pacific white shrimp. The test diets were prepared in the feed laboratory of Auburn University, Auburn, AL, USA using standard practices. In short, pre-ground dry ingredients and oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was then blended into the mixture to attain a consistency appropriate for pelleting. Each diet was pressure pelleted using a meat grinder and a 3 mm die. After pelleting, diets were dried to a moisture content of 8-10% and stored at 4 C.

Experimental system

For all the trials, Pacific white shrimp were stocked into 75-L tanks which were a component of a 2,500-L indoor recirculation system (reservoir, bead filter, fluidized biological filter and circulation pumps and heater). Each diet was offered to shrimp in three to five replicate aquaria for the length of the trial. Water temperature was maintained at around 28 C using a submerged 3,600-W heater (Aquatic Eco-Systems Inc., Apopka, Florida, USA). Dissolved oxygen was maintained near saturation using air stones in each aquarium and the sump tank using a common airline connected to a regenerative blower. Dissolved oxygen and water temperature were measured twice a day using a YSI-55 digital oxygen/temperature meter (YSI corporation, Yellow Springs, Ohio, USA) while pH, TAN and Nitrite-N measured once per week.

Free amino acids (AA) of hemolymph

To determine levels of free amino acids (AA) in the hemolymph of shrimp, replicate groups of four shrimp were utilized for each collection point. Shrimp at a given time point were netted and placed in an ice slurry to anesthetize the shrimp, after which hemolymph was collected from the abdominal segments using a syringe and 25 gauge needle.

For this work a 1 ml syringe was preloaded with 0.5 ml of an anti-coagulant and used to collect approximately 0.5 ml of hemolymph. To maintain semi-quantitative data, trial one recorded the volume whereas in the following trials, the weight of the anticoagulant and hemolymph were determined gravimetrically. The anticoagulant solution (Liu *et al* 2004) contains 30 mM Sodium Citrate Tribasic Dihydrate (Sigma S4641); 0.34 M Sodium Chloride (NaCl); 10 mM EDTA – Ethylene Diamine Tetraacetic Acid (Sigma, E9884); in de-ionized (DI) water. This is made by weigh 4.4115 g sodium citrate tribasic dehydrate, 9.945 g sodium chloride, 1.461 g EDTA, and dissolve in 500 ml DI water. To obtain free amino samples of the hemolymph, the collected hemolymph was centrifuged and decanted. The blood samples from shrimp in a single aquarium were pooled resulting in 2 to 3 samples/treatment each with blood from 3-4 shrimp.

Three trials were conducted with the Basal Diet (BD) as well as the BD with select AA. For each trial, the shrimp were preconditioned to the diets for four days. The prior evening the tanks were cleaned to remove any food sources. The next day and prior to feeding a group of shrimp were bled to determine fasted levels of free AA, the remaining shrimp were offered food for predetermined time periods and then the feed was withdrawn and shrimp fasted.

Growth Trials

A series of growth trials were carried out using juvenile shrimp. Diets were offered using a standard feeding protocol with 4 feedings per day. Diets were offered to shrimp at a suitable level based on historic performance as well as observed consumption of the feed. At the conclusion of the growth trials, the shrimp were weighed, enumerated, and frozen for subsequent analysis.

Biochemical analysis

Samples of hemolymph and shrimp were freeze until required. Tissue samples requiring analysis were sent to University of Missouri laboratory.

Statistical analysis.

All data was analyzed using SAS (V9.4. SAS Institute, Cary, NC, USA). Data from growth trials were analyzed using one-way analysis of variance to determine significant ($P < 0.05$) differences among the treatment means followed by Student-Neuman-Keuls multiple range test to distinguish significant differences between treatment means.

Results and Discussion

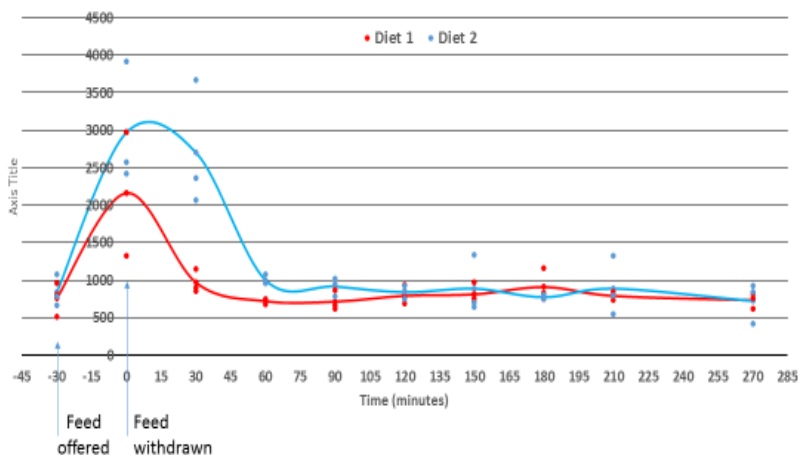
Due to the limitation of available data on methionine requirements for shrimp, initial work concentrated on confirming amino acid levels in the hemolymph of shrimp. A small component of this data is presented which demonstrates the upswings after feeding and the clearance of

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amino acids once the shrimp were fasted (Figures 1 – 3). We have evaluated both long term (up to 4.5 hours after feeding) as well as short term (while feeding).

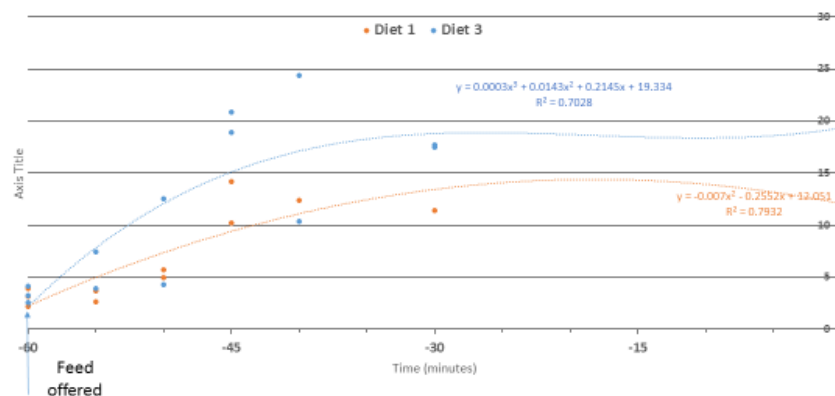
In the first trial presented in this summary, the shrimp were fasted overnight to allow the identification of basal levels of AA. After overnight fasting the shrimp were sampled (-30 minutes), then provided feed for 30 minutes after which another sample was taken (0 minutes) and the post feeding levels of AA determined (0 to 270 minutes). As most AA followed a similar pattern, the sum of AA in the hemolymph is presented in Figure 1. It is clear that all AA were near their peak after feeding and the level declined within 60 minutes to near fasting levels. Albeit, AA levels were followed over 270 minutes for which there was no secondary peaks for methionine or the other supplemented amino acids (lysine, arginine or taurine).

Figure 1. Shrimp (7 g) were offered one feeding over a 30 minute period after fasting overnight. Total AA levels ($\mu\text{g/ml}$) of hemolymph is presented for Diets 1 (Basal) and Diet 2 (Basal +Lys, Meth, Arg, Taur).



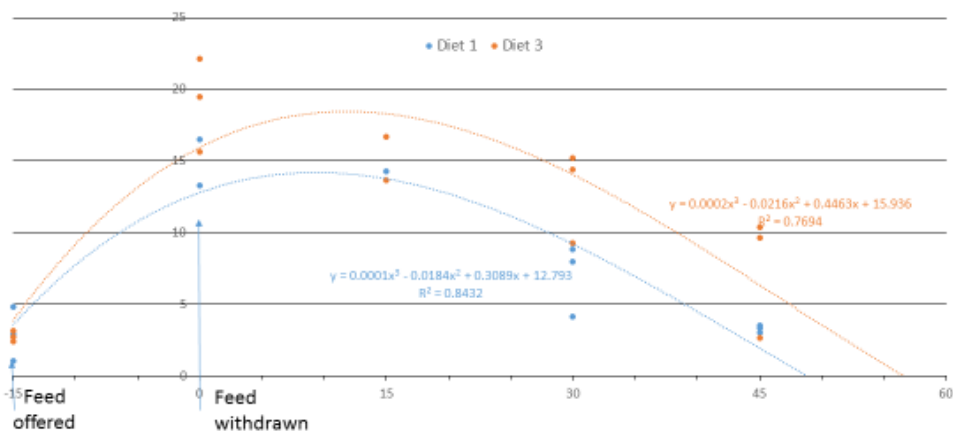
To better define the absorption process and to evaluate if there may be an asynchronous peak during absorption, a second trial was conducted, which simply looked at the uptake of AA over a 1 hr period (Figure 2). Again, the shrimp were fasted overnight and their hemolymph sampled to determine basal levels (-60 minutes). The shrimp were then offered feed for a total of 60 minutes during which time intermittent samples were taken. This data is presented for methionine (Figure 2), albeit the trend was similar for all amino acids. Both the supplemented basal (Diet 1) and DL-methionine supplemented diets (Diet 3) were followed. Although, the level of AA is consistently higher in the supplemented diet, the pattern for AA uptake is similar with a clear upswing in AA levels in 10-15 minutes after the initiation of feeding. This is quite different from that of fish species for which digestion and absorption is delayed several hours primarily due to holding of food in the stomach.

Figure 2. Shrimp (30.6 g) were offered one feeding after fasting overnight. Methionine levels ($\mu\text{g}/\text{ml}$) of hemolymph over a 60 minute period when feed is available to the shrimp is presented for Diets 1 (Basal) and 3 (Basal + DL methionine).



The third trial focused on the reduction of AA levels post feeding identify if there are different patterns that would indicate asynchronous absorption. For this trial, the shrimp were fasted overnight and a basal AA level determined (-15 minutes) the shrimp were then allowed to have feed for 15 minutes at which time a sample was taken (0 minutes) and the feed withdrawn. Samples were collected after 15, 30 and 45 minutes. To confirm the basal levels, an additional sample was taken after ~1.5 hr (data not presented). The methionine levels of the hemolymph are presented in Figures 3 with other amino acids following a similar pattern. Once again, the supplemented diet has higher levels of AA but there was no clear asynchronous uptake or clearance from the hemolymph.

Figure 3. Methionine levels (ug/ml) levels of hemolymph from shrimp (16.6 g) fed Diets 1 (Basal) and Diet 3 (Basal + DL methionine). Shrimp were sampled after overnight fasting (T-15) and offered feed for 15 minutes (T-15 to T0) then fasted over a 60 minute period.



The presented data, clearly demonstrates that AA in shrimp is much quicker than that of fish species with AA appearing in the hemolymph within 10 to 15 minutes. This is primarily due to the rapid and continual processing of food by shrimp. Based on the results of these studies there is no evidence that shrimp cannot utilize free AA or that there is asynchronously absorbed.

Presently there are few studies published on methionine requirement of the Pacific white shrimp. The few studies dealing with methionine supplements have failed to establish what would be considered a strong and convincing methionine requirement for this species. Hence, the second component of the research was designed to evaluate the efficacy of various methionine sources as well as attempt to identify a dietary requirement. Table 1a presents a series of diets which were supplemented with a range of methionine sources including: DL-methionine, coated methionine, as well as a modified diet using intact proteins. Proximate and AA composition of the test diets are presented in Table 1b with the basal diet analyzing at 0.49% methionine (0.41 cysteine) and the remaining diets at 0.64 to 0.68% methionine. These diets were offered to 0.5 g shrimp over a 42-day growth trial after which the AA profile of the shrimps tail muscle and hepatopancreas were evaluated. Growth and survival were very good with over 1000% weight gain observed with good FCR and survival (Table 1c). However, there were no differences in the growth, feed or survival rates observed. To determine if there were shifts in tissue composition, AA levels of the shrimp muscle and hepatopancreas were determined. There were no differences or trends seeing in methionine levels of these tissues. These results indicated that there were no differences in deposition across all treatments.

Table 1a. Composition (g/100g as is) of test diets.

	Coated			Intact protein
	Basal	Meth (76%)	DL-Met	
Poultry by product meal	4.00	4.00	4.00	4.00
Soybean meal	48.0	48.00	48.00	33.00
Corn protein concentrate	0.00	0.00	0.00	15.00
Gelatin	5.00	5.00	5.00	0.00
Menhaden fish oil	5.68	5.68	5.68	5.59
Lecithin	1.00	1.00	1.00	1.00
Cholesterol	0.08	0.08	0.08	0.08
Corn Starch	3.68	3.64	3.69	8.26
Whole wheat	27.00	27.00	27.00	27.00
Mineral premix	0.50	0.50	0.50	0.50
Vitamin premix	1.80	1.80	1.80	1.80
Choline chloride	0.20	0.20	0.20	0.20
Stay C 35% active	0.10	0.10	0.10	0.10
CaP-dibasic	2.80	2.80	2.80	3.00
Lysine				0.47
Methionine sources		0.20	0.15	0.00
Glycine	0.16			

Table 1b. Analysis of test diets.

	Basal	Coated Meth	DL-Met	Intact protein
Crude Protein	34.56	34.89	34.91	35.87
Moisture	5.85	5.87	5.4	5.23
Crude Fat	9.64	10.07	10.12	9.22
Crude Fiber	3.68	3.88	3.91	3.37
Ash	6.49	6.28	6.31	6.16
Amino Acids				
Alanine	1.72	1.72	1.73	2.01
Arginine	2.30	2.32	2.33	1.88
Aspartic Acid	3.06	3.08	3.11	2.84
Cysteine	0.41	0.42	0.42	0.53
Glutamic Acid	6.10	6.16	6.19	7.12
Glycine	2.69	2.53	2.53	1.46
Histidine	0.74	0.74	0.75	0.78
Hydroxy-lysine	0.11	0.10	0.11	0.05
Hydroxy-proline	0.65	0.64	0.65	0.12
Isoleucine	1.34	1.34	1.34	1.49
Lanthionine	0.03	0.02	0.01	0.02
Leucine	2.31	2.32	2.34	3.63
Lysine	1.83	1.84	1.86	1.83
Methionine	0.49	0.67	0.67	0.64
Phenylalanine	1.52	1.52	1.54	1.85
Proline	2.32	2.34	2.36	2.47
Serine	1.39	1.43	1.45	1.54
Taurine	0.22	0.22	0.22	0.23
Threonine	1.12	1.13	1.14	1.18
Tryptophan	0.38	0.38	0.37	0.36
Tyrosine	1.02	1.04	1.04	1.37
Valine	1.50	1.51	1.52	1.64
Sum AA	33.25	33.47	33.68	35.04

Table 1c. Response of juvenile shrimp (0.55 ± 0.04 g) to various test diets over a 42-day growth trial. Means of three replicate groups of shrimp.

Diet	Final Biomass (g)	Mean Weight (g)	Weight Gain (g)	Percent Gain	FCR	Survival (%)
Basal	65.99	6.60	6.03	1064.3	1.63	100.0
Coated	68.49	6.85	6.29	1127.9	1.56	100.0
DL-Met	66.04	6.60	6.07	1134.4	1.62	100.0
Intact	63.58	6.99	6.45	1197.6	1.53	90.0
P value	0.9533	0.7205	0.704	0.6533	0.217	0.5251
PSE	5.179	0.2384	0.2622	59.77	0.7118	4.71

To determine if we could induce a response to graded levels of methionine the first dose response trial utilized both DL-methionine and a microencapsulated methionine (Table 2a). This trial was repeated twice with the data from the second trial reported. The analysis for proximate and AA composition of the test diets is presented in Table 2b. The methionine levels ranging from 0.54% (0.42% cysteine) in the basal diet to 0.83% methionine for the DL-methionine supplemented diet and 0.81% methionine for the microencapsulated methionine diet. Diet 10 was designed as an intact protein diet utilizing corn protein concentrate which increased the methionine to 0.62% of the diet.

At the conclusion of a 42-day growth trial (Table 2c) the shrimp grew from 0.21 g to a maximum weight of 6.26g. Survival and growth were very good in this trial and there was a large increase in weight gain or tissue replacement (2467 to 2832%). However, the response was not typical of a dose response study. That is to say there was no increase in weight gain corresponding to increases in methionine levels. Proximate composition and AA profile of the whole shrimp were also evaluated for this trial with a slight decrease in the level of protein in the shrimp and significant differences in methionine levels of the whole shrimp. In this trial, the basal diet resulted in a significant depression in whole body tissue levels that was lower than most other diets. Based on regression analysis, there is a general increase as the level of methionine increased from 0.54% of the diet, albeit not a very strong response ($R^2 = 0.54$). This may indicate a slight deficiency of the basal diet.

Table 2a. Composition (g/100g as is) of test diets

	DL- Methionine					Micro-encapsulated methionine				
	1	2	3	4	5	6	7	8	9	10
Poultry by product meal	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Soybean meal	47.20	47.20	47.20	47.20	47.20	47.20	47.20	47.20	47.20	47.20
Menhaden Fish Oil	5.70	5.70	5.70	5.70	5.70	5.70	5.70	5.70	5.70	5.55
Corn Starch	2.83	2.83	2.83	2.83	2.83	2.78	2.73	2.63	2.53	2.08
Whole wheat	27.09	27.09	27.09	27.09	27.09	27.09	27.09	27.09	27.09	27.09
Trace Mineral premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Stay C	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
CaP-dibasic	2.20	2.20	2.20	2.20	2.20	2.20	2.20	2.20	2.20	2.20
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Corn protein isolate										7.90
DL-Methionine		0.05	0.10	0.20	0.30					
Meth. Micro-encapsulated						0.10	0.20	0.40	0.60	
Gelatin	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	
Glycine	0.30	0.25	0.20	0.10		0.25	0.20	0.10		0.30

Table 2b. Proximate and amino acid (AA) composition of test diets (g/100g as is)

Exp1615	1	2	3	4	5	6	7	8	9	10
Crude Protein	37.76	38.05	37.97	37.54	37.5	38.03	37.84	37.93	38.24	37.68
Moisture	5.13	6.48	6.47	7.41	7.42	6.65	7.07	6.61	6.76	5.89
Crude Fat	9.32	8.62	8.86	8.79	8.64	9.22	9.29	10.03	9.21	9.58
Crude Fiber	3.12	3.72	3.7	3.71	3.54	3.83	3.77	3.72	3.72	3.76
Ash	5.91	5.83	5.75	5.72	5.72	5.75	5.74	5.72	5.79	5.84
Amino Acid										
Alanine	1.91	1.94	1.88	1.92	1.93	1.94	1.93	1.93	1.95	1.92
Arginine	2.45	2.56	2.58	2.54	2.55	2.54	2.57	2.57	2.59	2.23
Aspartic Acid	3.21	3.33	3.28	3.29	3.29	3.33	3.31	3.33	3.36	3.32
Cysteine	0.42	0.42	0.42	0.42	0.42	0.43	0.42	0.43	0.42	0.53
Glutamic Acid	6.39	6.5	6.41	6.42	6.44	6.5	6.46	6.49	6.55	7.22
Glycine	3.29	3.26	3.08	3.11	3.06	3.26	3.21	3.12	3.04	1.95
Histidine	0.76	0.78	0.77	0.77	0.77	0.78	0.78	0.78	0.79	0.86
Hydroxy-lysine	0.12	0.14	0.13	0.14	0.14	0.13	0.13	0.13	0.13	0.07
Hydroxy-proline	0.86	0.87	0.83	0.95	0.97	0.86	0.87	0.87	0.88	0.16
Isoleucine	1.38	1.43	1.42	1.4	1.39	1.42	1.4	1.4	1.43	1.61
Lanthionine	0.01	0.04	0.02	0.03	0.03	0.03	0.02	0.02	0.02	0.06
Leucine	2.39	2.45	2.45	2.41	2.4	2.44	2.42	2.43	2.46	3.34
Lysine	1.92	1.98	1.96	1.95	1.95	1.98	1.97	1.97	1.99	1.81
Methionine	0.54	0.6	0.63	0.73	0.83	0.61	0.63	0.75	0.81	0.62
Phenylalanine	1.58	1.62	1.63	1.6	1.6	1.61	1.61	1.62	1.63	1.89
Proline	2.66	2.53	2.56	2.58	2.6	2.57	2.62	2.62	2.65	2.28
Serine	1.51	1.53	1.46	1.51	1.51	1.55	1.51	1.53	1.53	1.59
Taurine	0.16	0.19	0.23	0.22	0.21	0.21	0.22	0.21	0.21	0.21
Threonine	1.17	1.2	1.18	1.18	1.19	1.2	1.19	1.2	1.2	1.29
Tryptophan	0.41	0.4	0.37	0.36	0.37	0.38	0.38	0.38	0.37	0.42
Tyrosine	0.99	1.1	1.12	1.09	1.09	1.08	1.11	1.11	1.11	1.38
Valine	1.57	1.61	1.6	1.59	1.58	1.6	1.59	1.59	1.63	1.75

Sum AA 35.7 36.48 36.01 36.21 36.32 36.45 36.35 36.48 36.75 36.51

Table 2c. Response of juvenile shrimp (0.21 ± 0.017 g) to various test diets over a 42-day growth trial. Means of four replicate groups of shrimp. These diets were evaluated in two trials, results of the second trial are presented. Means within the same column with different letters are significantly different based on Student-Newman-Kuels test. Dunnett's T-test did not indicate that any of the values within a column were different from that of the basal diet.

Trt.	Diet	Final No.	Final Biomass (g)	Mean Weight (g)	Weight Gain (g)	Weight Gain %	FCR	Percent Survival
1	Basal	15.0	85.70	5.71ab	5.49ab	2520.5	1.39ab	100.0
2	DL 0.05	15.3	81.70	5.36b	5.15b	2473.7	1.48a	101.7
3	DL 0.1	15.3	87.05	5.71ab	5.50ab	2591.3	1.39ab	101.7
4	DL 0.2	14.8	85.65	5.80ab	5.59ab	2582.6	1.37ab	98.3
5	DL 0.3	15.0	89.85	5.99ab	5.77ab	2623.9	1.32ab	100.0
6	Micro 0.1	15.0	85.80	5.72ab	5.51ab	2580.9	1.39ab	100.0
7	Micro 0.2	14.8	87.65	5.94ab	5.72ab	2696.3	1.34ab	98.3
8	Micro 0.4	15.5	84.10	5.43b	5.22b	2467.0	1.46a	103.3
9	Micro 0.6	14.8	86.30	5.85ab	5.64ab	2665.0	1.35ab	98.3
10	Intact Protein	15.0	93.85	6.26a	6.04a	2832.4	1.26b	100.0
P-value		0.3984	0.1303	0.0180	0.0169	0.7155	0.0193	0.3984
PSE		0.2261	2.584	0.160	0.1584	129.98	0.0395	1.507

The last growth trial the basal diet (Table 3a) was similar to that used in other trials and would be expected to have a similar level of methionine. The first series of diets were formulated with increasing levels of corn protein concentrate (0, 5, 10, 15, 20 and 25%) as a substitute for soybean meal allowing for an incremental increase in dietary methionine. In the second set of diets, the basal diet was supplemented with high levels of DL-methionine (0, 0.1, 0.2, 0.4, 0.6%). These diets were then offered to juvenile shrimp (0.85g) over a 42-day growth trial. In this trial, the shrimp offered the diets supplemented with DL-methionine and the highest level of corn protein concentrate were significantly smaller than the shrimp offered the basal diet. Again, there was not trend in the data in terms of improved growth due to methionine supplementations whether it be from intact protein or DL-methionine. Once again there is no consistency to the data, which points to the basal diet not being deficient or marginally deficient at best.

Table 3a. Composition (g/100g as is) of test diets

	Basal	CP5	CP10	CP15	CP20	CP25	M-0.1	M-0.2	M-0.4	M-0.6
Menhaden fishmeal	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50
Soybean meal	46.10	39.58	33.06	26.55	20.04	13.51	46.10	46.10	46.10	46.10
Corn protein concentrate		5.00	10.00	15.00	20.00	25.00				
Gelatin	5.00	4.00	3.00	2.00	1.00		5.00	5.00	5.00	5.00
Menhaden fish oil	5.43	5.49	5.55	5.61	5.67	5.73	5.43	5.43	5.43	5.43
Lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Corn Starch	3.69	6.15	8.61	11.06	13.51	15.98	3.69	3.69	3.69	3.69
Whole wheat	27.00	27.00	27.00	27.00	27.00	27.00	27.00	27.00	27.00	27.00
Mineral premix	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Stay C	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
CaP-dibasic	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Arginine			0.17	0.35	0.53	0.70				
Lysine			0.07	0.24	0.42	0.59				
DL-Methionine							0.10	0.20	0.40	0.60
Threonine	0.19	0.20	0.21	0.22	0.24	0.25	0.19	0.19	0.19	0.19
Glutamic acid	1.41	1.40	1.15	0.79	0.41	0.06	1.31	1.21	1.01	0.81

Table 3b Response of juvenile shrimp (0.85 g) to various test diets over a 42-day growth trial. Means of four replicate groups of shrimp is presented. Means with different superscripts within a column are significantly different based on Student-Newman-Kuels test. Means with a * indicates a significant difference in the response as compared to that of the basal diet.

	Final Biomass (g)	Mean Weight (g)	Weight Gain (g)	Weight Gain (%)	FCR	Percent Survival
Basal	53.8	5.98	5.14	611.3	1.92	90
CP5	44.89	4.99	4.14	483.7	2.46	90
CP10	42.87	4.72	3.89	470.3	2.58	90
CP15	52.23	5.51	4.63	533.2	2.16	95
CP20	46.49	5.41	4.59	565.4	2.17	87.5
CP25	35.44*	4.65*	3.79*	440.5*	2.71	77.5
M-0.1	41.33	4.44*	3.61*	432.1*	2.75*	92.5
M-0.2	38.48	4.66*	3.82*	455.0*	2.57*	82.5
M-0.4	45.78	5.72	4.86	566.2	2.04	80
M-0.6	52.34	5.6	4.72	539.3	2.08	93.33
P-value	0.047	0.0162	0.017	0.0256	0.0330	0.5178
PSE	4.065	0.3218	0.3212	38.83	0.1955	6.165

Conclusion

Research to date in our laboratory has demonstrated that the uptake of amino acids in shrimp corresponds to the digestive physiology of the animal, in that shrimp are semi-continuous feeders that process and digest foodstuffs very quickly. From the initiation of feeding, an upswing in amino acids in the hemolymph was apparent. The clearance of amino acids was also relatively fast with amino acid levels returning to overnight fasting levels within 60 minutes. This cycle of nutrient corresponds to the semi-continuous feeding habits of shrimp. Based on both absorption and clearance patterns of the amino acids, there was no indication of a synchronous absorption of supplemented amino acids.

Results from a series of growth trials conducted with a range of supplements as well as intact protein sources producing a range of dietary methionine levels yet consistent and persuasive data was not obtained. Across four independent growth trials, increasing the level of methionine as a pure supplement or using corn protein concentrate as an intact protein source, produced very little evidence of growth enhancement. Clearly, some of the issue is variation inherent in shrimp trials. This leads to three possible conclusions: 1) the basal diet is replete or only marginally deficient in methionine and TSAA, making the methionine supplementation ineffective and the identification of a deficiency more problematic, 2) another amino acid is first-limiting, thereby also rendering methionine supplementation ineffective, or 3) crystalline methionine does not work in shrimp feeds, though this is contradicted by the work conducted with lysine in which crystalline and intact lysine both produced a dietary response. Given the number of attempts as well as reports in the literature of poor responses to methionine levels once can only conclude that the deficiency is at or below that of our basal diets (0.54 methionine and 0.42% cysteine). This data does not correspond to reports in the literature which indicate a higher methionine requirement. It is this authors opinion, that we need a method and diet that will consistently and repeatedly induce a deficiency. Until this has been developed there will be no agreement on a dietary requirement.

Unfortunately, unless we can demonstrate a dose response to methionine (or other AA) levels, feed formulators are going to continue to over-formulate diets with fishmeal and will limit

Davis, A. and M. Duan. 2017. Fact or Fiction: Methionine Requirement for Pacific White Shrimp *Litopenaeus vannamei*. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. . (Eds), Investigación y Desarrollo en Nutrición Acuicola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 32-54.

the use of soybean meal. Hence, we propose to take a step back and develop a test diet with crystalline amino acids to evaluate the ranking of limiting amino acids. This approach will provide the foundation for a systematic evaluation of EAA requirements for the Pacific white shrimp.

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El Ciclo Diario de la Digestión en Peces Cultivados.

Aspectos Funcionales y Metodológicos

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Resumen

La eficiencia de la digestión depende entre otros factores de la adecuada relación entre enzima y sustrato, así como del tiempo de hidrólisis bajo las condiciones apropiadas. La digestión es un proceso temporal que, en peces, como en otros vertebrados, empieza con la detección del alimento y finaliza con la expulsión de los desechos. La disponibilidad del alimento en el medio es variable a lo largo del ciclo diario por lo que también lo es la ingestión y digestión de los nutrientes. Cabe pues esperar que haya momentos óptimos para suministrar el alimento y que favorezcan su digestión, y momentos más desfavorables en los que se reduciría la eficiencia de digestión. Por eso, el diseño de protocolos de alimentación más eficientes requiere del conocimiento previo de los ciclos digestivos. En esta revisión se describirán los estudios realizados en este sentido y el estado de conocimiento de la función digestiva en relación a los ciclos diarios. Se muestran tanto los avances en larvas de peces como en juveniles y adultos que presentan diferentes modos de digestión. Dada la variabilidad entre especies, y la escasez de estudios, la información disponible es aún muy fragmentaria para definir patrones de actuación.

Palabras clave: Peces, digestión, ritmos diarios, protocolo de alimentación

Introducción

Los protocolos de alimentación utilizados en la cría y engorde de peces son variados entre las diferentes instalaciones de cultivo, así como entre especies. Estos protocolos, con distinta ración y frecuencia de alimentación diarias, generalmente se han establecido en base a la propia experiencia previa y a abordajes empíricos y no están sustentados por un conocimiento de los fundamentos biológicos y tecnológicos. En la alimentación de peces, como en otros vertebrados, la eficiencia con que se incorporan los nutrientes al organismo depende entre otros aspectos de la interacción entre la disponibilidad del alimento y las capacidades digestivas de la especie. Como todo proceso fisiológico, la digestión tiene una dimensión temporal que se inicia por una serie de estímulos asociados a la presencia de alimento y su detección, y finaliza cuando los nutrientes ingeridos han sido convenientemente hidrolizados para permitir su absorción por las paredes intestinales y los desechos expulsados. En este proceso temporal, otro elemento a considerar es el tránsito digestivo que afecta al tiempo en que el alimento ingerido está disponible para ser hidrolizado por las enzimas digestivas condicionando la eficiencia digestiva. Es evidente que el nivel de disponibilidad del alimento en el medio no es permanentemente constante ni es igual de accesible para el organismo al que alimenta a lo largo del día. Por ello, la ingestión de alimento presenta igualmente un patrón diario con variaciones entre horas (Boujard & Leatherland 1992; Madrid, Boujard & Sánchez-Vázquez 2001) y cabe esperar por lo tanto que también suceda lo mismo con su posterior digestión.

A lo largo de la evolución, los organismos han adaptado sus pautas vitales a los ciclos naturales para optimizar los procesos fisiológicos frente a los cambios predecibles de los factores medioambientales. Para ello, las funciones vitales están controladas por un sistema de osciladores biológicos internos que permiten a los organismos estar preparados de forma anticipada a los acontecimientos cíclicos de la naturaleza dependientes de los movimientos de la Tierra en relación a los astros que la rodean (Pittendrigh 1993; Reebbs 2002). Especialmente influyente en la alimentación animal es el ciclo día/noche que influye en la presencia de alimento, su detección e ingesta. El sistema circadiano endógeno oscila en

ciclos cercanos a 24 horas en ausencia de señal externa y se sincronizan por estímulos externos, particularmente la luz (Whitmore, Foulkes y Sassone-Corsi 2000; Vatine, Vallone, Gothilf y Foulkes 2011). Aun considerando que la iluminación es un sincronizador preferente, la alimentación también presenta un papel destacado de forma independiente (Spieler 1992; Feliciano, Vivas, de Pedro, Delgado, Velarde y Isorna 2011; López-Olmeda 2017).

Se ha descrito que la mayoría de los peces se alimentan predominantemente utilizando la visión por lo que es necesario un determinado nivel de iluminación para que detecten el alimento, aunque también influye de manera destacada el olfato y hay especies en que la iluminación no es un factor necesario. Desde el punto de vista de la acuicultura, estas variaciones diarias en la actividad alimentaria, con máximos y mínimos a determinadas horas, nos llevan a pensar que existen ciertos momentos más apropiados para suministrar el alimento en los cuales se favorecería el proceso de digestión, y otros en los que la digestión no sería igual de eficiente, con el consiguiente descenso en la relación coste/beneficio del proceso de engorde y exceso de vertido de residuos nitrogenados al medio. Por ello, una buena planificación de los protocolos de alimentación fijando adecuadamente el momento y la frecuencia de las tomas diarias, tendría que tener en cuenta estos patrones diarios. Sin embargo, el conocimiento que disponemos de la función digestiva en peces está basado principalmente en muestreos puntuales en un determinado momento del día que no reflejan la situación periprandial ni cómo se organiza este proceso durante un ciclo de 24 horas.

Resulta curioso que mientras las variaciones de productos de la absorción intestinal o productos hormonales en plasma se han examinado profusamente en estudios de respuestas rítmicas y postprandial (Boujard y Leatherland 1992; Cowan, Azpeleta y López-Olmeda 2017), los cambios en la propia función digestiva no hayan recibido apenas atención en peces. A diferencia de lo que ocurre en crustáceos (Molina, Cadena y Orellana 2000; Nolasco-Soria y Vega-Villasante 2000; Espinosa-Chaurand, Vega-Villasante, Carrillo-Farnés y Nolasco-Soria 2017), hasta hace relativamente pocos años nuestro conocimiento sobre las variaciones diarias de la actividad de las enzimas digestivas como respuesta a la

ingestión era bastante escaso. Recientes estudios empiezan a darnos una idea de cómo se organiza la función digestiva para adaptarse a periodos repetitivos de 24 horas. Hay que tener en cuenta que cuando hablamos de la función digestiva no solo nos referimos a la actividad de las enzimas digestivas determinada con técnicas bioquímicas e indicadores de una cantidad determinada de enzimas en el tracto digestivo, sino también a los pasos previos que preparan la maquinaria enzimática. Los precursores de los enzimas están codificados por los correspondientes ARNm cuya expresión relativa también va a mostrar variaciones horarias.

Los peces constituyen un grupo de numerosísimas especies adaptadas a una extrema diversidad de hábitats. Debido a la variedad de hábitos de alimentación y comportamiento, así como morfológica y fisiológica, se pueden encontrar notables diferencias en el proceso de digestión entre grupos taxonómicos y especies. Las diferencias son incluso más notables cuando se comparan diferentes etapas de la vida, sobre todo durante el desarrollo y transformación de larvas a juveniles. Teniendo en cuenta todas estas fuentes de variabilidad la información disponible es aún bastante fragmentaria y dispar. Tomando como base nuestros estudios, en esta revisión se harán una puesta al día de nuestros conocimientos sobre la digestión en peces en el marco del ciclo diario y se describirán los aspectos más destacables de este proceso en las diferentes etapas del ciclo de vida, así como los problemas metodológicos asociados a este tipo de investigación.

Las primeras fases del desarrollo

Las larvas de peces son planctívoras y su alimentación en cultivo se basa en mantener una concentración mínima presas de forma permanente en los tanques de cultivo, lo que favorece la posibilidad de que se estén alimentando continuamente. Una práctica bastante habitual en larvicultura de peces es prolongar las horas de luz estableciendo generalmente fotoperiodos de iluminación permanente durante las primeras semanas después de la eclosión. Con esta práctica y considerando que las larvas de mayoría de las especies cultivadas son predatoras visuales se pretende favorecer la ingestión diaria y así acelerar el

crecimiento. La literatura existente sobre el comportamiento alimentario en larvas plantea ciertas dudas sobre la utilidad de esta práctica, ya que los ritmos de alimentación diarios también se han descrito en poblaciones de larvas mantenidas con iluminación permanente (Fujii, Kurokawa, Kawai, Yosedá, Dan, Kai, *et al.* 2007; Yosedá, Yamamoto, Asami, Chimura, Hashimoto & Kosaka 2008; Mata-Sotres, Martínez-Rodríguez, Pérez-Sánchez, Sánchez-Vázquez y Yúfera 2015). De aquí la necesidad de conocer con detalle los ciclos de digestión en estas primeras etapas.

En las larvas de peces la digestión tiene lugar en un ambiente neutro-alcalino en el intestino medio y distal, ya que el estómago sólo se desarrolla generalmente al final de esta fase. No obstante, también se ha descrito el desarrollo precoz del estómago en algunas especies (Rønnestad, Yúfera, Ueberschär, Ribeiro, Sæle & Boglione 2013). El comienzo de la alimentación es una fase crucial y bien definida en el desarrollo de peces (Yúfera & Darias 2007). Solo cuando todos los órganos relacionados con la captura de presas y con el tránsito digestivo están preparados y son funcionales se inicia la alimentación. Teniendo en cuenta que tanto la luz como el alimento son sincronizadores de los osciladores endógenos, un interesante desafío es saber si el comienzo de la alimentación deja alguna huella en los ciclos de alimentación. La información al respecto es vaga y heterogénea. Como se ha comentado anteriormente, en varias especies se ha observado que los ritmos de alimentación perduran en ausencia de alternancia luz/oscuridad, aunque pierden la sincronía con los teóricos día y noche, incluso pueden desaparecer en el transcurso del desarrollo (Dou, Seikai y Tsukamoto 2000). Igualmente se ha descrito en algunas especies que los ritmos de expresión de los genes reloj en condiciones de iluminación permanente desaparecen con el progreso desarrollo (Davie, Sanchez, Vera, Sánchez-Vázquez y Migaud 2011; Martín-Robles, Whitmore, Pendón & Muñoz-Cueto 2013). No obstante, en dorada (*Sparus aurata* (L.)), las variaciones circadianas del nivel de ingestión y de la expresión de los genes reloj se mantuvieron al menos durante dos meses en condiciones de luz permanente (Mata-Sotres *et al.* 2015). Además, en este estudio se observó que los ciclos detectados en larvas a los pocos días de iniciar la alimentación estaban ya desfasados con respecto a las que se mantuvieron con un ciclo día/noche (Fig. 1). Esto puede deberse a que

el momento de la primera ingestión, usualmente en las primeras horas de la tarde, podría haber determinado la acrofase de los ciclos. Confirmando así, que el alimento también es sincronizador de los ritmos en larvas.

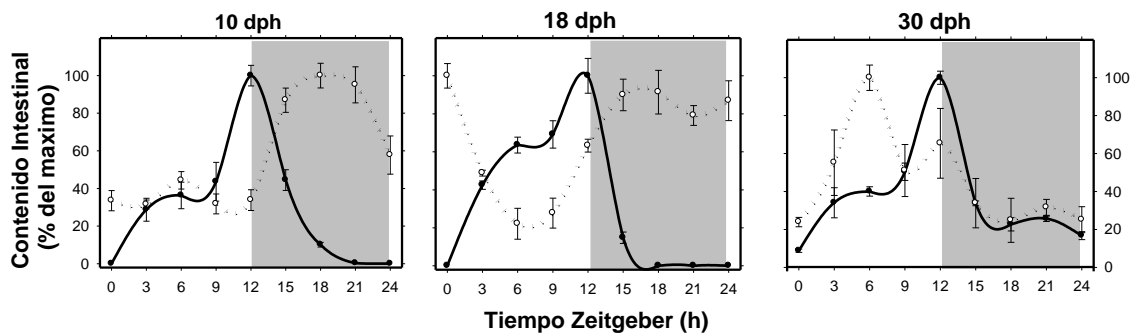


Figura 1. Patrones diarios del contenido del tubo digestivo en larvas de *Sparus aurata* a diferentes días desde la eclosión (dph). Línea continua: con fotoperiodo de 12h Luz/12h Oscuridad. La zona sombreada indica el periodo de oscuridad. Línea punteada: con luz continua. (Modificado de Mata-Sotres *et al.* 2015).

En cualquier caso, el comienzo de la alimentación es gradual, tanto en cantidad de alimento ingerido como en la proporción de larvas que están alimentándose, hasta que se detecta alimento en el 100% de las larvas, periodo que difiere entre especies. Es bien conocido que las larvas de peces están preparadas para digerir desde el primer momento, ya que disponen del ARNm que codifican los enzimas y de zimógenos en el páncreas desde antes de la apertura de la boca (Zambonino-Infante y Cahu 2001; Lazo, Darias & Gisbert 2011). Sin embargo, la información de cómo se inicia la digestión con la primera ingesta de alimento es prácticamente inexistente y se limita a un estudio con larvas de mero, *Epinephelus malabaricus* (Bloch & Schneider), (Fujii *et al.* 2007) y otro con larvas de lenguado, *Solea senegalensis* (Kaup), (Navarro-Guillén, Moyano, y Yúfera, 2015), una especie que presenta una fase de vida y alimentación pelágica durante dos semanas hasta que acaba la metamorfosis. En dichos estudios se observa que la actividad de la tripsina ya presenta un ritmo diario desde el primer día de ingestión. Por el contrario, no se observaron claros ciclos diarios en la actividad de otros enzimas digestivos en *S. senegalensis* (Navarro-Guillén *et al.* 2015).

Una vez comenzada la alimentación y con todas las larvas alimentándose de forma rutinaria y activa se puede observar que bajo condiciones naturales de iluminación la actividad alimentaria muestra marcados ciclos adaptados a la alternancia de luz/oscuridad. Esto se ha observado tanto en el medio natural como en poblaciones en cultivo (Okauchi, Oshiro, Kitamura, Tsujigado y Fukusho 1980; Mackenzie, Ueberschär, Basford, Heath y Gallego 1999; Shoji, Maehara y Tanaka 1999; Dou *et al.* 2000; Østergaard, Munk y Janekarn 2005; Kotani y Fushimi 2011). Así, en especies como *Paralichthys olivaceus* (Temminck & Schlegel), *Melanogrammus aeglefinus* (L.), *Acanthopagrus schlegeli* (Bleeker), *Scomberomorus niphonius* (Cuvier), se han descrito claros ciclos de ingestión con preferencias a alimentarse en los crepúsculos, amanecer y atardecer, si bien el patrón diario de ingestión parece tener un alto componente específico. En algunas especies como *Misgurnus anguillicaudatus* (Cantor), *Cynoglossus semilaevis* (Günther) y *S. senegalensis* se ha descrito un cambio gradual de preferencias diurnas a nocturnas cuando se pasa de la fase pelágica a bentónica después de la metamorfosis (Ma, Liu, Xu, Liang y Zhuang 2006; Wang, Hu, Wang, Cao, Yang, Lü, *et al.* 2008; Navarro-Guillén *et al.* 2015). En experimentos más detallados realizados con larvas de *S. aurata* y *S. senegalensis*, se ha observado que en presencia continua de alimento y con fotoperiodo de luz/oscuridad, las larvas van aumentando el contenido intestinal durante la fase de luz alcanzando el máximo al iniciarse la fase oscura para evacuar el contenido progresivamente durante dicha fase oscura (Mata-Sotres *et al.* 2015; Navarro-Guillén *et al.* 2015). Este incremento no es continuo y suele presentar dos fases ingestivas preferentes, una por la mañana y otra por la tarde, que se acumulan y resultan en un incremento casi en continuo del contenido intestinal durante la fase diurna. En postlarvas de lenguado después del inicio de la fase bentónica tras la migración ocular, la alimentación es prácticamente continua durante todo el ciclo diario completo tanto de día como de noche. Finalmente, los juveniles de esta especie mostrarán una actividad netamente nocturna (Bayarri, Muñoz-Cueto, López-Olmeda, Vera, Rol de Lama, Madrid, *et al.* 2004).

En cualquier caso, los patrones diarios de la función digestiva en larvas de peces están muy poco estudiados. A excepción de los estudios de Ueberschär (1993; 1995) con muestras oceánicas de larvas de *Clupea harengus* (L.), *Sardina pilchardus* (Walbaum) y *Sprattus sprattus* (L.), de Mackenzie *et al.* (1999) con muestras oceánicas de *M. aeglifinus*, y de Fujii *et al.* (2007) en larvas cultivadas de *E. malabaricus* sobre la actividad trípica, solo recientemente se han realizado determinaciones continuas de algunas enzimas pancreáticas en un par de especies mantenidas en el laboratorio, *S. senegalensis* y *S. aurata* (Navarro-Guillén *et al.* 2015; Mata-Sotres, Moyano, Martínez-Rodríguez y Yúfera 2016). En estos estudios cada enzima digestiva muestra un patrón de actividad particular. En el caso de las proteasas, solo la actividad de la tripsina se ha examinado en detalle. En todos los casos se ha encontrado un ritmo claro de la actividad trípica y mostrando una alta correlación con la actividad alimentaria, hasta el punto de que a veces se observan patrones casi paralelos al contenido digestivo (Fig. 2). Esta parece ser una respuesta general ya que se ha descrito en todos los estudios mencionados anteriormente. Esta respuesta no es sorprendente si tenemos en cuenta que la tripsina está considerada como una enzima digestiva crucial en larvas de peces (Ueberschär, 1993) necesaria para movilizar la elevada cantidad de aminoácidos que hacen falta para la generación de nuevos tejidos en organismos con una tasa de crecimiento muy elevada. En especies de agua templadas, el tiempo de residencia del alimento en el intestino larvario está limitado a 1 a 2 horas (Yúfera, Fernández-Días y Pascual 1995; Andrade, Brazão, Nogueira, Ferreira, Dillinger, Dinis, *et al.* 2011; Ortiz-Monis, Mancera y Yúfera 2017), y la hidrólisis proteica se tiene que realizar con rapidez para maximizar la biodisponibilidad de los aminoácidos y su pronta incorporación a los tejidos corporales (Morais, Lacuisse, Conceição, Dinis y Rønnestad 2004).

Por el contrario, los patrones de la actividad diaria de lipasas, y amilasas no muestran claros ritmos diarios. Tanto en *S. aurata* como en *S. senegalensis* las actividades se varían con ciertas oscilaciones durante el periodo diario y solo en ocasiones se observa un descenso al final del periodo de oscuridad que se recupera al iniciarse el periodo de luz (Mata-Sotres *et al.* 2015; Navarro-Guillén *et al.* 2015). Hasta donde sabemos no hay estudios en larvas de otras especies sobre la evolución diaria completa de estas enzimas.

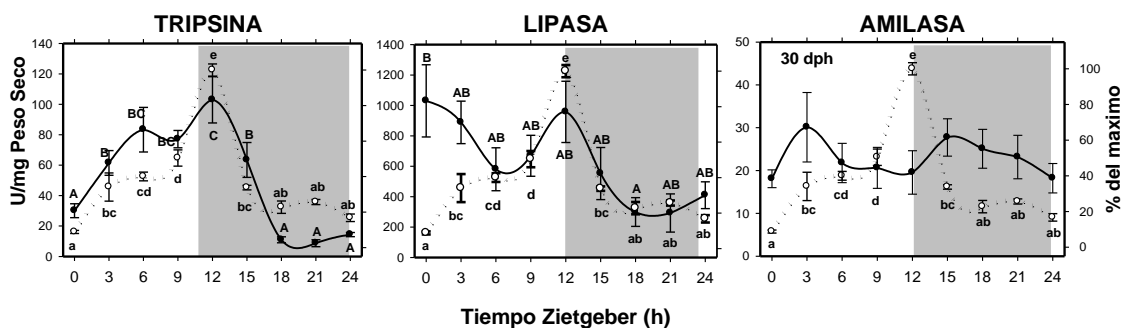


Figura 2. Patrones de actividad diaria de tripsina, lipasa y amilasa pancreáticas en comparación con el contenido del tubo digestivo en larvas de *Sparus aurata* de 30 días desde la eclosión con fotoperiodo de 12h Luz/12h Oscuridad. Línea continua: actividad enzimática. Línea punteada: contenido del tubo digestivo. La zona sombreada indica el periodo de oscuridad. (Modificado de Mata-Sotres *et al.*, 2016).

Las variaciones diarias de la expresión del ARNm de los principales enzimas pancreáticos (*tripsinógeno*, *chimotripsinógeno*, *fosfolipasa A2*, *lipasa activada por sales biliares*, y *α -amilasa*) se han analizado en larvas de *S. aurata* (Mata-Sotres *et al.* 2016). En este estudio se ha observado que los patrones diarios difieren notablemente del de las actividades descritas en el mismo experimento. Esta diferencia es esperable puesto que la expresión molecular está regulada de forma diferente a la actividad bioquímica. También hay que tener en cuenta que las isoformas enzimáticas que contribuyen a la actividad de un determinado enzima no tienen por qué corresponder plenamente con la isoforma del transcrito que ha sido clonado y con el que se ha medido la expresión génica. De forma general, la expresión de estas enzimas suele ser menor en las primeras horas del periodo de oscuridad y más elevada al final del periodo de oscuridad y/o al inicio del periodo de luz. Es decir el ARNm de las enzimas digestivas tiende a sobre expresarse cuando se va a iniciar el siguiente ciclo de alimentación de manera que la maquinaria estaría preparada para sintetizar los enzimas necesarios. Una respuesta anticipatoria que permitiría una mejor utilización de los nutrientes ingeridos.

Desde el punto de vista práctico resulta interesante comparar los resultados entre larvas alimentadas con presas vivas o con microdietas. En un experimento con larvas de *S. aurata*

(Mata, Moyano, Martínez-Rodríguez & Yúfera 2014.) se comprobó los patrones de ingestión eran similares en larvas alimentadas en continuo con rotíferos y con una elevada frecuencia con microdieta, en ambos casos mostrando la respuesta de incremento bi-modal antes mencionada. También los patrones de tripsina eran muy semejantes, pero no el de la actividad de lipasa que alcanzaba valores muy inferiores con esta microdieta experimental. Sin embargo, las expresiones génicas de tripsinógeno y pre-lipasa activada por sales biliares si fueron muy similares. Estos resultados indican que la regulación digestiva por efecto de la dieta ocurre a nivel post-transcripcional y en este caso en particular se pone en evidencia una limitación en la digestión de la fracción lipídica de la microdieta.

Juveniles y adultos

Tras la adquisición de los caracteres definitivos del sistema digestivo, el proceso de hidrólisis de los nutrientes ingeridos presenta unos rasgos bien diferenciados de la fase larvaria. En peces con estómago es la aparición de las glándulas gástricas y funcionalidad de este órgano el que define el final de esta transición a juvenil desde el punto de vista de la alimentación. El engorde de peces se realiza generalmente con iluminación natural o condiciones que simulan la alternancia de día-noche. El suministro del alimento en juveniles y adultos suele ser más puntual con una o varias comidas durante el día. El tránsito digestivo está controlado en parte por el estómago que actúa como contenedor y distribuidor de la ingesta al resto del tubo digestivo. La digestión tiene dos fases claramente diferenciadas y sucesivas, una digestión gástrica en ambiente ácido (excepto en peces agástricos) y posteriormente una digestión intestinal en ambiente neutro-alcalino. Por tanto, las condiciones iónicas en el lumen del tubo digestivo constituyen un factor clave en la activación de los correspondientes enzimas. Existen diversos estudios que caracterizan los intervalos óptimos de pH y temperatura para la actividad de los enzimas digestivos en peces. Hay ciertas variaciones entre especies pero de forma general la pepsina está activa entre 0,5 y 4 con un óptimo cercano a pH 2, mientras que la tripsina lo hace entre pH 7 y 12 (Alarcón, Díaz, Moyano & Abellán 1998; Wang, Wang, Wang, Xue y Sun 2006; Nalinanon, Benjakul, Visessanguan y Kishimira 2008; Chen, Cao, Yoshida, Liu, Weng,

Sun, *et al.* 2009). Así pues, la tripsina puede estar activa en un intervalo más amplio que la pepsina. La cuestión que se plantea es si estos valores de pH se dan en el lumen gastrointestinal de juveniles y adultos, y en tal caso, durante cuánto tiempo. Las mediciones secuenciales de pH gastrointestinal en peces teleósteos se limitan a una decena de especies cubriendo en mayor o menor medida el periodo postprandial.

En vertebrados se han descrito dos estrategias básicas de acidificación gástrica. Por una parte, especies que mantienen una acidificación permanente en el estómago; por otra parte especies que mantienen un ambiente luminal neutro entre comidas, y la acidificación gástrica solo se inicia al entrar el alimento en el estómago recuperándose los valores neutros al vaciarse (Secor 2003; Papastamatiou & Lowe 2005). La mayoría de los peces teleósteos estudiados muestran la segunda estrategia (Deguara, Jauncey y Agiuss 2003; Yúfera, Fernández-Díaz, Vidaurreta, Cara y Moyano 2004; Nikolopoulou, Moutou, Fountoulaki, Venou, Adamidou & Alexis 2011; Yúfera, Moyano, Astola, Pousão-Ferreira y Martínez-Rodríguez, 2012; Márquez, Robles, Morales & Moyano 2012; Rosero 2013; Hlophe, Moyo y Ncube 2014; Solovyev, Kashinskaya, Rusinek y Izvekova 2016; Jacob, Pouil, Lecchini, Oberhänsli, Swarzenski y Metian 2017; Yúfera, Nguyễn y Trần 2017), aunque también se han descrito una acidificación permanente en un par de especies como la trucha arcoíris, *Oncorhynchus mykiss* (Walbaum) (Sugiura, Roy y Ferraris 2006; Bucking & Wood 2009), y la cobia, *Rachycentrum canadum* (L.) (Yúfera, Nguyen, Engrola, Conceição, Jordal, Le, *et al.* 2016). En el intestino siempre se ha descrito un ambiente neutro-alcalino, variando entre una ligera acidificación y una ligera alcalinización. Lo relevante de estos patrones iónicos es cómo afectan a la actividad de las enzimas. Considerando que en las especies que acidifican el estómago después de la ingestión la pepsina sólo se activa a valores inferiores a pH 4, la actividad de la pepsina dependerá del patrón postprandial del pH gástrico y de que se realmente se alcancen los niveles mínimos de activación, no que no siempre ocurre (Fig. 3). Por otra parte, en especies que presentan una acidificación permanente puede haber una subida de pH gástrico motivada por el efecto tampón y de dilución de la ingesta pudiendo quedar por encima de los pH óptimos de activación de la pepsina (Bucking & Wood 2009).

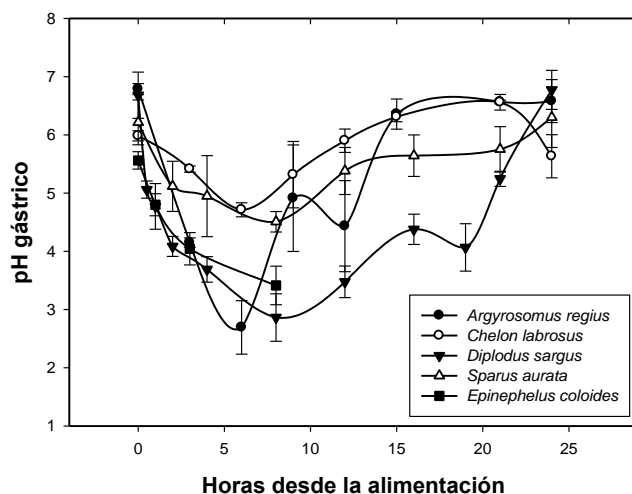


Figura 3. Variación postprandial del pH gástrico (media y error estándar) en varias especies de teleósteos (recopilado de Yúfera *et al.* 2012, 2014, 2017 y Rosero 2013).

La proteólisis ácida se realiza con la participación de la pepsina que se activa desde su precursor (pepsinógeno) cuando el pH es suficientemente bajo, y de la bomba de protones, que es la responsable de verter el CLH que acidificará en lumen del estómago. Un detalle metodológico importante a este respecto es como se analiza la actividad pepsina. La metodología usual se basa en una reacción de hidrólisis tamponada a pH 2 (Anson 1938), por lo que el resultado es indicativo de la cantidad de pepsina teóricamente disponible; es decir pepsina activa + pepsinógeno. Pero como se ha comentado anteriormente, en peces no siempre se dan estos valores tan bajos de pH gástrico (Fig. 3 y 5) por lo que es más apropiado tamponar la reacción al pH luminal medido en la muestra si queremos tener una estimación más realista de la actividad de la pepsina en ese momento del ciclo. La actividad postprandial de proteasas tanto ácida como alcalina en juveniles y adultos se ha estudiado en muy pocas especies. Para comprender el patrón básico de respuesta digestiva postprandial lo más adecuado es observar que ocurre en la situación más sencilla, peces que reciben una sola comida diaria ofrecida siempre a la misma hora. En este caso el estómago se llena rápidamente y tras un lapso de tiempo se evacua lentamente durante varias horas pasando al intestino (Nikolopoulou *et al.* 2011; Yúfera *et al.* 2012; Márquez *et al.* 2012).

Por su parte, el intestino muestra un patrón similar, pero con cierto desplazamiento en el

tiempo. La actividad de la pepsina muestra valores dispares según los estudios. En experimentos con *Diplodus sargus* (L.) y *S. aurata*, cuando se ha utilizado el pH luminal real en la determinación analítica, se observa un aumento de la pepsina tras la ingestión que desciende cuando el estómago se está vaciando (Yúfera *et al.* 2012, 2014) (Fig. 4 y 5). Cuando la pepsina se ha analizado tamponando a un pH cercano a 2 se ha encontrado una actividad constante o casi constante en *D. sargus*, *S. aurata* y *Anguilla anguilla* (L.) (Montoya, López-Olmeda, Yúfera, Sánchez-Muros y Sánchez-Vázquez 2010; Yúfera *et al.* 2012; López-Olmeda, López-García, Sánchez-Muros, Blanco-Vives, Aparicio y Sánchez-Vázquez 2012), aunque también se han encontrado picos de actividad no relacionados con el momento de la ingestión (Yúfera *et al.* 2014). Esto indica que el pepsinógeno estaría permanentemente disponible y solo se activaría al descender el pH por la acción de la bomba de protones.

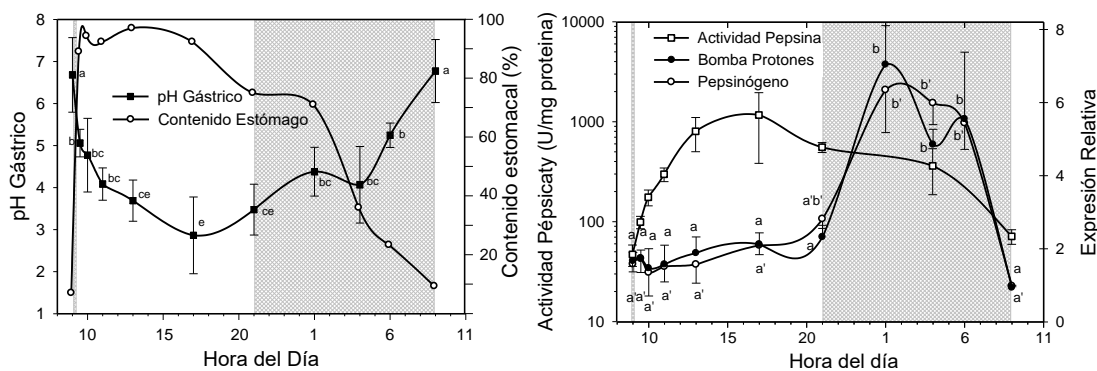


Figura 4. Respuesta digestiva gástrica en juveniles de *Diplodus sargus* frente a una única comida matutina (9:00 h). Izquierda: Llenado y vaciado del estómago y evolución del pH luminal. Derecha: Actividad de la pepsina medida al correspondiente pH gástrico y expresión génica del pepsinógeno y la bomba de protones. La zona sombreada indica el periodo de oscuridad.

Con respecto a proteasas alcalinas (tripsina y chimotripsina) los estudios realizados en *O. mykiss* (Santigosa, Sánchez, Médale, Kaushik, Pérez-Sánchez y Gallardo 2008), *S. aurata* (Montoya *et al.* 2010; Morales, Márquez, Saenz de Rodríguez, Bermúdez, Robles y Moyano 2014), *S. senegalensis* (Rodiles, Santigosa, Herrera, Hachero-Cruzado, Cordero, Martínez-Llorens *et al.* 2012), *A. anguilla* (López-Olmeda *et al.* 2012), *Silurus*

meridionalis (Zeng, Fu, Li, Li, Li, Cao *et al.* 2014) y *Dicentrarchus labrax* (L.) (Castro, Couto, Pérez-Jiménez, Serra, Díaz-Rosales, Fernandes *et al.* 2016) muestran ciertas discrepancias en el momento en que actividad se incrementa, que puede ser antes o, más habitualmente, algún tiempo después de la ingestión. De hecho, los máximos de actividad se pueden alcanzar bastantes horas después de la ingestión, esto concordaría con el tiempo necesario para el quimo pase al intestino. Otros enzimas como lipasas y amilasas muestran igualmente máximos de actividad varias horas después de la ingestión y no necesariamente coincidentes con los máximos de tripsina (Santigosa *et al.* 2008; Montoya *et al.* 2010; López-Olmeda *et al.* 2012).

Estos patrones se modifican sensiblemente cuando se complica el protocolo de alimentación. En experimentos en los que la hora de alimentación varió aleatoriamente de día a día se ha observado la actividad de la proteasa alcalina y amilasas puede ser constante o presentar cierto aumento postprandial adaptándose a la hora de ingesta (Vera, De Pedro, Gomez-Milán, Delgado, Sánchez-Muros, Madrid, *et al.* 2007; Montoya *et al.* 2010).

Las discrepancias se deben probablemente a aspectos metodológicos relativos a la talla del pez, condiciones previas al experimento, el tipo y cantidad de alimento, y al número de muestras postprandiales que se han tomado. Todo esto dificulta enormemente la comparación de resultados, sobre todo cuando se intenta establecer una respuesta general a un protocolo de alimentación diario estable. De hecho, es difícil discriminar si un incremento de actividad enzimática en las horas previas a la alimentación se debe a una respuesta anticipatoria o por el contrario a una respuesta tardía dependiente del tiempo de tránsito requerido para alcanzar el intestino medio.

De igual manera, la ampliación de la frecuencia de alimentación a dos o más comidas diarias altera los perfiles de acidificación gástrica, y en consecuencia los de la actividad enzimática de la pepsina. En un estudio con juveniles tempranos de *S. aurata* (Yúfera *et al.* 2014) se observó que, en los peces alimentados dos veces al día, la pepsina mostraba un máximo varias horas después de cada suministro de alimento. Sin embargo, cuando el

alimento se suministró en continuo la actividad péptica mostraba un solo máximo diario, al igual que cuando se le suministró una sola vez. Otro aspecto destacable en este estudio es que los valores máximos de actividad de pepsina se incrementaron con la frecuencia de alimentación, alcanzando 5, 14 y 40 U/g de pez respectivamente con 1 comida, 2 comidas y en continuo durante la fase diurna (Fig. 4).

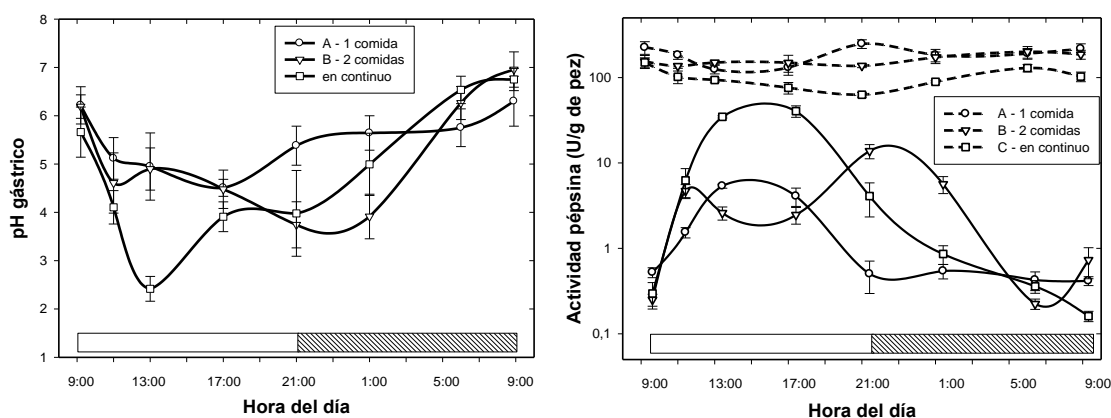


Figura 4. Variación del patrón diario del pH gástrico (izquierda) y actividad péptica (derecha) en juveniles de *Sparus aurata* alimentados con diferentes frecuencias diarias (A: una comida a las 9:00 h; B: dos comidas, a las 9:00 y 16:00 h; C: en continuo durante la fase diurna. En línea continua se muestra la actividad péptica determinada al correspondiente pH luminal. En línea punteada se muestra la actividad determinada a pH 2. (modificado de Yúfera *et al.* 2014).

La información sobre expresión génica de los correspondientes precursores enzimáticos es aún más escasa. [En](#) Solo en juveniles de *D. sargus* se ha encontrado una respuesta evidente de incremento paralelo de la expresión los genes implicados en la digestión ácida (pepsinógeno y de la bomba de protones) cuando se ha vaciado el estómago y cesado la actividad de la pepsina (Yuferá *et al.* 2012). En este caso la expresión fue elevada durante todo el periodo nocturno descendiendo justo antes de la siguiente comida, lo que sugiere que se estaría reponiendo el pepsinógeno necesario para el siguiente ciclo digestivo. En juveniles de *S. aurata*, los resultados no fueron tan claros. Sólo se detectó un aumento de expresión durante la transición de la noche al día en peces alimentados una sola vez o en continuo (Yúfera *et al.* 2014). También en juveniles de *O. mykiss* se ha observado un incremento del ARNm del pepsinógeno unas 6 horas después de la alimentación, cuando

aun hay bastante contenido estomacal (Borey, Panserat, Surget, Cluzeaud, Plagnes-Juan, Herman *et al.* 2016). Otro aspecto interesante de este estudio, es que el incremento de la expresión de enzimas intestinales como prolidasa, fosfolipasa y maltasa se detectó a las dos horas de la alimentación, pero con un máximo 12 horas después, cuando el quimo llega al intestino. En este caso, parece ser que tanto la ingestión como el tiempo de tránsito digestivo están modulando la respuesta génica.

Como se puede ver, la información disponible en la fase de engorde es heterogénea y aún insuficiente para establecer patrones generales de respuesta digestiva. Quizás las diferencias fisiológicas inter-específicas pesen mucho en estas diferencias. Lo que sí parece deducirse de todos estos experimentos es que, independientemente de la frecuencia de alimentación, en peces aclimatados a un protocolo de alimentación diario de rutina, el tubo digestivo tiende a estar vacío antes de la primera comida del día siguiente, y la digestión se organiza en periodos de 24 h. Cuando se pasa de una sola a varias comidas diarias las respuestas postprandiales se solapan y finalmente parecen acomodarse en patrones específicos para cada protocolo de alimentación. Seguramente, los cambios temporales de actividad están encaminados a economizar energía y maximizar la eficiencia digestiva bajo las condiciones rutinarias de alimentación específicas a las que se encuentran sometidos los peces.

Consideraciones finales y conclusiones

Es evidente que el conocimiento sobre los ciclos diarios de digestión en peces es aún muy incompleto. Los resultados disponibles proceden de un pequeño número de especies y en diferentes condiciones experimentales. Hay otras fuentes de variación además de las descritas aquí. Existe un cambio de talla manifiesto entre los juveniles después de la metamorfosis y los adultos que han alcanzado la madurez sexual; no hay información de cómo afectaría esto al tránsito y los ciclos digestivos. En algunas especies se ha descrito una dicotomía en el comportamiento alimentario en una misma especie entre diurnos y nocturnos, esto se refleja obviamente en los ciclos de digestión. Además, generalmente solo se ha examinado en unas pocas enzimas digestivas, dando prioridad a las proteasas, y no

hay un estudio profundo de todas las enzimas pancreáticas e intestinales que permitan obtener una visión de conjunto. Aún hay menos información a nivel molecular, por lo tanto, es difícil vislumbrar aspectos clave de regulación de la respuesta anticipatoria.

Por todo esto, ni siquiera en una determinada especie tenemos un barrido mínimamente completo de diferentes posibilidades de alimentación que contribuya a comprender como cambia la función digestiva cuando se modifica el régimen de alimentación. En todo caso, sí se han producido avances que permiten ver ciertas respuestas. Como se ha descrito anteriormente, los perfiles de actividad diaria varían sensiblemente al cambiar los horarios y frecuencia de alimentación. Esta adaptación de la función digestiva a unas condiciones de rutina plantea un interesante dilema; si debemos adaptar los protocolos de alimentación a los ritmos digestivos, o los ritmos se adaptan por si solos a los diferentes protocolos establecidos. Los presentes resultados indican que hay unos límites en esta adaptación, y que la misma cantidad de alimento diario se ingiere y dirige mejor si está repartido en más de una toma al día. El tener la posibilidad de elegir el momento preferente para alimentarse, ya sea por alimentación continua o con alimentadores a demanda, parece rendir los mejores resultados. Pero para asegurar esto aún hacen falta estudios de digestibilidad que acompañen a los diferentes protocolos.

La dificultad logística de muestreos periódicos continuados durante 24 o 48 horas es una de las razones de la escasez de este tipo de estudios. El número de muestras necesarias para la obtención de patrones fiables puede disminuir sensiblemente el número y densidad de peces en los tanques de experimentación. En experimentos de nutrición y engorde el número de peces es un factor crítico y la disminución drástica de la población a mitad del experimento puede afectar sensiblemente al resultado final. Por otra parte, un muestreo persistente puede afectar al estrés de los peces y resultar en valores atípicos y no representativos en los últimos puntos del muestreo.

Lo que está claro es que sólo con muestreos periódicos durante el ciclo diario se puede obtener una idea representativa de la capacidad y respuesta digestiva a un determinado protocolo; una sola muestra diaria proporciona en muchos casos una visión errónea de la

realidad ya que el máximo de actividad se puede alcanzar a diferentes horas según el protocolo de alimentación.

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Aditivos Alimentarios para Camarones Marinos: Salud y Nutrición

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Resumen

El sector de la camaronicultura viene enfrentando dificultades relativas al surgimiento de enfermedades. Para la prevención de mortalidades, el uso profiláctico de fármacos quimioterapéuticos es una práctica común. Sin embargo, debido al residuo dejado en la carne de los animales y la selección de bacterias resistentes, el uso de antibióticos en la acuicultura fue prohibido en varios países. Por lo tanto, es necesario el desarrollo de estrategias alternativas para el control de enfermedades en el cultivo de camarones, tales como el uso de probióticos, prebióticos, ácidos orgánicos y compuestos bioactivos. Los probióticos son bacterias benéficas, que al ser suministradas colonizan el tracto digestivo de los animales de cultivo con el objetivo de mejorar la salud de estos animales. Los probióticos actúan en la microbiota del hospedero inhibiendo el crecimiento de bacterias patógenas, ya sea por la producción de compuestos antimicrobianos o por exclusión competitiva. Los prebióticos son ingredientes alimentares no digeribles que afectan benéficamente el hospedero estimulando selectivamente el crecimiento y/o la actividad de un determinado grupo de bacterias benéficas en el tracto digestivo. Otro aditivo que viene ganando espacio en la industria del cultivo de camarones son los ácidos orgánicos o sus sales. Los ácidos orgánicos o sus sales ejercen efectos sobre el desempeño zootécnico de los animales durante los cultivos inhibiendo bacterias patógenas en el tracto intestinal del camarón. Las macroalgas también pueden ser usadas como aditivos alimentares para camarones ya que tienen actividad antimicrobiana frente a bacterias y poseen compuestos que pueden actuar como inmunoestimulantes para camarones.

Palabras claves: Probióticos, prebióticos, ácidos orgánicos, macroalgas.

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1. Introducción

La producción acuícola mundial creció progresivamente en las últimas décadas a una tasa media anual del 3,2 por ciento, alcanzando una producción de 106 millones de toneladas en 2015 (FAO-FIGIS 2017) y superando la captura (93,7 millones de toneladas). Además, la producción mundial de camarones marinos en cautiverio, alcanzó la marca de 4,8 millones de toneladas producidas en 2015 (FAO 2017). El camarón blanco del pacífico, *Litopenaeus vannamei*, es la especie más cultivada siendo responsable del 79,5% del volumen total producido (FAO 2017).

Sin embargo, la intensificación de los cultivos, la contaminación y los disturbios ecológicos y alimentarios llevaron al surgimiento de enfermedades en el cultivo de camarones (Defoirdt *et al.* 2011) principalmente de orígenes virales y bacterianos, resultando en pérdidas importantes en los cultivos. En 2012 y particularmente en 2013 los volúmenes de producción de cultivo de camarón disminuyeron como resultado de la aparición de diferentes enfermedades, como el síndrome de mortalidad precoz (EMS) causada por una cepa de *Vibrio parahaemolyticus*, en algunos países de Asia y América Latina (Tran *et al.* 2013).

Las infecciones causadas por bacterias del género *Vibrio* se consideran un problema importante en el cultivo de camarones, teniendo como síntomas anorexia, inactividad, baja tasa de crecimiento, necrosis muscular y, por consiguiente, mortalidad (Chiu *et al.* 2007). Los antibióticos se han utilizado comúnmente como tratamiento contra las vibriosis. Aunque algunos de estos productos pueden disminuir la incidencia de mortalidad, el mal uso en la acuicultura ha llevado a la aparición de bacterias resistentes (Defoirdt *et al.* 2011). Además, existe una preocupación sobre los impactos potenciales que los residuos químicos puedan causar en la salud humana y el medio ambiente. Debido a esto, la Unión Europea ha prohibido el uso de antibióticos en la producción animal, siendo esta una tendencia mundial.

Por lo tanto, medidas para proteger los cultivos sin uso de antibióticos están siendo probados y adoptados por productores del mundo entero como el uso de aditivos alimentarios, que además de efecto en la salud, actúan en la nutrición, auxiliando en la mejora del desempeño de los camarones. En este escenario, destacamos los probióticos,

prebióticos, ácidos y sales orgánicas y derivados de macroalgas pardas, cuyos conceptos y usos en la carcinicultura marina se abordarán en este capítulo.

2. Probióticos

2.1. Definición

Elie Metchnikoff es considerado el primer investigador en trabajar con el concepto de probióticos (Fuller 1992). Describió los probióticos como "microbios ingeridos con el objetivo de promover la buena salud". Esta misma definición fue modificada posteriormente por Fuller (1992) para "un suplemento alimenticio microbiano que afecta benéficamente al animal hospedero mejorando su balance microbiano intestinal".

Estas definiciones se aplicaron originalmente a animales terrestres. Pero para la acuicultura esta definición puede ser insuficiente, pues en ambientes acuáticos hay una constante y mayor interacción entre los organismos cultivados y los microorganismos presentes en el ambiente. Teniendo esto en cuenta, Gatesoupe (1999) define los probióticos para la acuicultura como "células microbianas que se suministran de tal manera que entren en el tracto gastrointestinal y que sean capaces de mantenerse vivas, mejorando la salud de los animales".

Igualmente, Gram *et al.* (1999) ampliaron la definición eliminando la restricción a la mejora sólo del intestino: "un suplemento microbiano vivo que afecta benéficamente al animal hospedero mejorando su balance microbiano". Y posteriormente, la FAO / WHO (2001) define los probióticos como "microorganismos vivos que al ser suministrados en cantidades adecuadas, confieren beneficios para la salud del hospedero".

Según Fuller (1992), estos microorganismos vivos deben cumplir algunas características esenciales para ser utilizados como probióticos: 1) Ser efectivos en la mejora de la salud del hospedero, 2) No ser tóxicos o patógenos, 3) Deben ser viables y capaces de sobrevivir al metabolismo digestivo 4) Ser capaces de colonizar el epitelio intestinal y de mantenerse estables durante un largo período de tiempo y 5) Poseer estabilidad en condiciones de almacenamiento y de campo.

2.2. *Mecanismo de acción de los probióticos*

Hay varias teorías que explican el modo de actuación de los probióticos en el equilibrio de la microbiota intestinal, en la mejora de la digestión de nutrientes y en la estimulación del sistema inmune.

2.2.1. *Equilibrio de la microbiota intestinal*

La flora bacteriana del tracto intestinal es un recurso natural completo que puede ser utilizado en un esfuerzo para reducir el impacto de bacterias patógenas en los cultivos. En animales acuáticos la microbiota intestinal está compuesta principalmente por bacterias gram negativas como *Vibrio*, *Pseudomonas*, *Aeromonas* o *Plesiomonas*, dependiendo de las condiciones del medio acuático. Sin embargo, estas bacterias pueden ser patógenas en condiciones de estrés, tanto en peces como en camarones.

Los probióticos han demostrado tener la capacidad de reducir las poblaciones de bacterias patógenas modificando la microbiota intestinal, permitiendo la colonización de bacterias benéficas y consecuentemente mejorando la salud del hospedero (Gatesoupe 1999)

Las bacterias benéficas pueden competir por nutrientes y / o espacio y son capaces de producir diferentes compuestos inhibitorios, sirviendo como antagonistas de bacterias patógenas. Esta capacidad de antagonismo es una de las principales propiedades a tener en cuenta a la hora de elegir un probiótico. Estudios ya demostraron la capacidad *in vitro* de inhibición de varios probióticos frente a diferentes patógenos (Balcázar *et al.* 2007a; Vieira *et al.* 2010). Esta capacidad inhibitoria se debe a la producción de compuestos como ácidos orgánicos, peróxido de hidrógeno, sideróforos y / o bacteriocinas (Vázquez *et al.* 2005).

Según Vázquez *et al.* (2005) el poder de inhibición de bacterias ácido-lácticas utilizadas como probióticos contra patógenos de peces es principalmente debido a la producción de ácido láctico y acético. Por otro lado, bacterias como *Pseudomonas fluorescence* poseen la capacidad de inhibir el crecimiento del patógeno *Vibrio anguillarum* por competición por minerales como el hierro (Gram *et al.* 1999).

Además de la producción de compuestos inhibitorios y de la competencia por nutrientes, los probióticos pueden competir por lugares de adhesión a los lugares de fijación por fuerzas pasivas, por interacciones electrostáticas, hidrofóbicas y estéricas, ácidos lipoteicoicos y por estructuras específicas de adhesión. La capacidad de adhesión de algunos probióticos como las bacterias ácido lácticas, ya ha sido comprobada por Balcázar *et al.* (2007a) donde demostraron que estas bacterias disminuyeron la adhesión de bacterias patógenas en la mucosa intestinal de la trucha.

Así, la adhesión y colonización de las superficies mucosas junto con la producción de compuestos inhibitorios son posibles mecanismos contra patógenos a través de una competencia por sitios de unión y nutrientes.

2.2.2. Mejora de la digestión de nutrientes

Los probióticos también pueden auxiliar en la digestión, ayudando al hospedero en la absorción o producción de nutrientes. Después de adherirse al tracto intestinal, el probiótico necesitará carbohidratos para su crecimiento y comenzará a producir enzimas extracelulares como proteasas, lipasas, amilasas y carbohidrasas, que pueden participar en la digestión de nutrientes en los camarones (Ochoa-Solano & Olmos-Sotos 2006, Wang *et al.* 2007). Esta producción enzimática puede aumentar la digestibilidad de ingredientes y también ayudar en la pre-digestión de nutrientes de origen vegetal presentes en la ración, mejorando parámetros zootécnicos, como crecimiento y eficiencia alimenticia.

La estimulación de la actividad enzimática por probióticos en camarones ha sido reportada por Wang *et al.* (2007). Este estudio comprobó que el probiótico *Bacillus* sp. mejoró el crecimiento y la actividad enzimática en *L. vannamei*, recomendando su uso para estimular la producción del cultivo.

Del mismo modo, un estudio con *L. vannamei* evidenció una mayor actividad de amilasa cuando los camarones fueron alimentados con el probiótico *Bacillus* sp. (Yu *et al.* 2009), y un otro estudio sugiere que el uso de probióticos en larvas de *L. vannamei* añadidos al agua en ciertas concentraciones, puede aumentar la actividad de algunas enzimas e incrementar la supervivencia (Zhou *et al.* 2009). Por lo tanto, la modificación de la microbiota intestinal por bacterias probióticas puede ser una fuente suplementaria de

alimento ofreciendo vitaminas y aminoácidos a través del aumento de la actividad enzimática.

2.2.3. Estimulación del sistema inmune

La microbiota normal del tracto intestinal influye en el sistema inmune del organismo formando parte de una red de protección inmunológica y no inmunológica, otorgando protección contra patógenos o tolerancia contra bacterias comensales presentes en el epitelio (Sanz & Palma 2009). Por lo tanto, al colonizar el tracto intestinal, los probióticos no sólo inhiben patógenos como también son capaces de estimular el sistema inmunológico del hospedero por poseer en sus paredes celulares lipopolisacáridos, peptidoglucanos y β -glucanos actuando como moléculas señalizadoras para activar el sistema inmune (Akhter *et al.* 2015).

Los camarones como invertebrados, carecen de un sistema inmune específico. Por lo tanto, su sistema de defensa está basado en un sistema inmune innato para reconocer a los patógenos invasores. El sistema inmunológico de los crustáceos está íntimamente relacionado con su sangre o hemolinfa y con las células circulantes, los hemocitos. Los hemocitos además de estar involucrados en la fagocitosis, también son responsables de la producción de melanina a través del sistema pro-fenoloxidasa que es un componente importante en la reacción de la defensa celular (Barraco *et al.* 2008).

El sistema inmunológico puede ser estimulado para mejorar la resistencia frente a diferentes enfermedades. En camarones, bacterias probióticas como *L. plantarum* es capaz de inducir la modulación del sistema inmune de *L. vannamei* tras infección con *V. alginolyticus* e *V. harveyi* aumentando su resistencia (Chiu *et al.* 2007; Vieira *et al.* 2010). Igualmente, camarones que recibieron *Streptococcus phocae* como probiótico tuvieron mayores conteos de hemocitos totales, mayor actividad de la enzima fenoloxidasa (PO) y mayor actividad fagocítica (Pattukumar *et al.* 2014).

2.3. Bacterias usadas como probióticos y sus efectos en la acuicultura

Existe una gran variedad de probióticos utilizados tanto en el cultivo de peces como de camarones. Las bacterias utilizadas comúnmente en la acuicultura pueden ser bacterias gram negativas o gram positivas.

2.3.1. Bacterias Gram negativas

Dentro del grupo de las bacterias gram negativas utilizadas como probióticos, las bacterias más comunes pertenecen a los géneros *Aeromonas*, *Pseudomonas* y *Vibrios*.

El género *Aeromonas* posee capacidad inhibitoria *in vitro* por producción de bacteriocinas frente a varios patógenos de importancia en la acuicultura. Estudios han confirmado esta capacidad inhibitoria *in vivo* en truchas, donde la suplementación con *Aeromonas* aumentó su resistencia frente a la infección con *Yersinia ruckeri* (Abbas *et al.* 2010), al igual que ayudó a aumentar la sobrevivencia total en el cultivo de *Artemia franciscana* (Gunasekara *et al.* 2010).

Cepas de *Pseudomonas* también poseen capacidad de inhibición frente a diferentes patógenos que afectan los cultivos de camarones. Como ejemplo, cepas de *Pseudomonas marina* son capaces de producir compuestos que inhiben el crecimiento de vibriones patógenos de camarón como *Vibrio harveyi*, *Vibrio fluvialis*, *V. parahaemolyticus*, *Vibrio damsela* y *Vibrio vulnificus* (Chythanya *et al.* 2002). Ya *in vivo*, el uso de *Pseudomonas* sp. en el cultivo aumentaron la resistencia de *Penaeus monodon* al ser desafiados con *V. harveyi* (Pai *et al.* 2010).

Por otro lado, especies de *Vibrio* al ser utilizadas como probiótico pueden entrar a competir con otras bacterias del cultivo inhibiendo posibles patógenos del sistema ayudando a mejorar la salud del camarón, como también pueden ayudar a aumentar la resistencia frente a infección por otros *Vibrios* patógenos como *V. parahaemolyticus* (Balcázar *et al.* 2007b).

No obstante, según Kesacordi-Watson *et al.* (2012), el uso de bacterias gram negativas está sujeto al riesgo de transferencia de material genético causando resistencias o virulencias.

2.3.2. Bacterias Gram positivas

Las bacterias gram positivas más utilizadas como probióticos pertenecen al grupo de las bacterias ácido-lácticas y al género *Bacillus* (Akhter *et al.* 2015). Las bacterias del género *Bacillus* se caracterizan por producir endosporas en situaciones adversas en el medio ambiente lo que les confiere una ventaja de presentar mayor viabilidad a lo largo del tiempo.

Diversas especies de *Bacillus* se han utilizado como probióticos: *B. subtilis*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus licheniformis* y *Bacillus coagulans* (Cutting 2011). Estas bacterias son capaces de producir diferentes metabolitos secundarios como antibióticos y enzimas, inhibiendo patógenos y ayudando en la digestión de nutrientes.

Según Ochoa-Solano y Olmos-Sotos (2006), diversas cepas de *Bacillus* aislados de sedimentos marinos poseen la capacidad *in vitro* para degradar proteínas y otros compuestos derivados de la soja, así como la capacidad de producir enzimas que degradan los carbohidratos y los lípidos. Adicionalmente, cepas aisladas del cultivo de camarón son capaces de reducir poblaciones de *Vibrio* spp. totales del sistema de cultivo y mejoran la respuesta hemato-inmunológica del camarón (Ferreira *et al.* 2015) como también mejoran la calidad del agua de cultivo ayudando en la degradación de la materia orgánica del sistema (Song *et al.* 2011).

En la acuicultura, las especies de *Bacillus* son muy utilizadas en formulaciones comerciales de raciones por la capacidad de producir compuestos inhibitorios y enzimas, y por la facilidad de incorporación en las raciones debido a que sus esporas les confieren mayor resistencia. Sin embargo, fue comprobado que cepas de *B. subtilis* fueron responsables de causar lesiones en la cutícula de *P. monodon* (Wang *et al.* 2000) y más recientemente, un estudio describió una nueva enfermedad llamada WPD (del inglés, White Patch Disease) en el camarón *L. vannamei* causada por *B. cereus* causando manchas blancas opacas en el caparazón, necrosis, coloración azul blanquecina, pérdida de apetito, músculos pálidos y mortalidad (Velmurugan *et al.* 2015).

Por otro lado, tenemos a las bacterias ácido-lácticas que constituyen un grupo de bacterias catalasa-negativas, no esporuladas, en forma de cocos o bacilos y producen ácido láctico como principal producto final durante la fermentación de los carbohidratos. Son de hábitat anaeróbico, pero pueden ser aeróbicos facultativos y ácido tolerantes. Generalmente están asociadas con hábitats ricos en nutrientes. Sin embargo, pueden estar presentes en la flora nativa de la boca, intestino y tracto urinario de mamíferos. Las delimitaciones del grupo han sido objeto de algunas controversias, sin embargo los géneros *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Enterococcus* y *Streptococcus* forman parte de este grupo (Carr *et al.* 2002).

La clasificación de las bacterias ácido lácticas en diferentes géneros está basada en gran medida en la morfología, en el modo de fermentación de la glucosa, en el crecimiento a diferentes temperaturas, en la configuración del ácido láctico producido, en la capacidad para crecer en altas salinidades, y en su tolerancia a la acidez o alcalinidad (Carr *et al.* 2002).

Las bacterias ácido-lácticas además de producir diferentes compuestos antimicrobianos, también se caracterizan por replicarse fácilmente y por estimular la respuesta inmune no específica del hospedero (Carr *et al.* 2002; Balcázar *et al.* 2007a), desempeñando así un papel importante en el control de enfermedades.

En algunos peces, estudios han asociado a las bacterias ácido-lácticas como parte de su microbiota normal. Ya en camarones, las bacterias ácido lácticas no son dominantes, sin embargo, cuando son adicionadas en la alimentación pueden incrementar su población en el intestino, disminuyendo la prevalencia de *Vibrio* spp., aumentando la respuesta inmune frente a infección con patógenos y consecuentemente aumentando la sobrevivencia (Vieira *et al.* 2010, Bolívar-Ramírez *et al.* 2013).

3. Prebióticos

Los prebióticos son definidos como ingredientes alimentarios no digeribles (fibras), que afectan benéficamente al hospedero, estimulando el crecimiento y la actividad de un determinado número de bacterias en el intestino. Para que un compuesto pueda ser considerado prebiótico, él tiene que cumplir algunos presupuestos como: resistir a la hidrólisis ácida y enzimática del tracto intestinal, ser un sustrato selectivo para el crecimiento de bacterias benéficas, induciendo a efectos benéficos en la salud del animal (O'sullivan *et al.* 2010).

Los prebióticos sirven como fuente de energía para las bacterias intestinales pudiendo ser llamado de sacáridos. Existen algunos trabajos, en vertebrados, que sugieren la acción de un inmuno-sacárido que actúa de forma directa en el sistema inmune innato, sin la necesidad de los probióticos (Kocher, 2004). Sin embargo, los prebióticos no son necesariamente inmuno-estimulantes y vice-versa. La actividad inmuno-moduladora de los prebióticos es mediada por las interacciones con los receptores de reconocimiento de patrones (PRR) de las células, los cuales son expresados en macrófagos que ligan a

receptores de transducción de señal como NF- κ B (factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas) que estimulan la respuesta inmune (Song *et al.* 2014). Este sacárido también puede interactuar con PRRs en la forma de microorganismo asociados patrones moleculares (mAmps), tales como proteína glucosilada o el polisacárido capsular de bacterias, desencadenar una respuesta inmune (Song *et al.* 2014). No obstante, en crustáceos, que carecen de sistema inmune adaptativo, todavía no está bien definido como los prebióticos pueden actuar en la modulación del sistema inmunológico. De esta forma, el prebiótico puede actuar de dos maneras: a través de la estimulación directa del sistema inmune innato, o a través del aumento del crecimiento de la flora microbiana del tracto intestinal (Song *et al.* 2014).

Los prebióticos son encontrados en azúcares absorbibles o no, fibras, péptidos, proteínas, alcoholes de azúcares y oligosacáridos (Barata, 2012). Los prebióticos más estudiados y utilizados en la acuicultura son los oligosacáridos como el fructooligosacárido (FOS), glucooligosacárido (GOS), mananoligosacárido (MOS) y los polisacáridos, como la inulina y las β -glucanos (Song *et al.* 2014).

Existen algunos estudios con prebióticos en camarón. Ramirez *et al.* (2013) evaluaron los efectos de la suplementación de 5 g kg⁻¹ de inulina administrada individualmente y en conjunto con el probiótico. En este trabajo fue observado que los efectos simbióticos de la inulina con el probiótico son capaces de cambiar la microbiota gastrointestinal, y aumentar la respuesta inmune frente al *Vibrio alginolyticus*. Sin embargo, la inulina sola no aumentó la resistencia a este patógeno y tampoco el crecimiento del camarón *L. vannamei*. Zhang *et al.* (2012) y Cuong *et al.* (2013) utilizando 4 g kg⁻¹ de mananoligosacárido (MOS) en concentrado de camarón marino *L. vannamei*, observaron una disminución en la concentración de *Vibrios* spp en la microflora intestinal, aumento en el incremento de peso semanal del camarón y mayor resistencia al estrés por amonio frente al desafío experimental con *Vibrio harveyi*, respectivamente.

3.1. Mananoproteína (MP)

Las MOS, cuando ligadas a una proteína, forman un complejo llamado de Mananoproteína (MP) (Morales-López *et al.* 2009). Por lo tanto, la MP es un prebiótico, derivado de la pared celular de la levadura (PCL) *Saccharomyces cerevisiae*. La PCL es

compuesta por tres partes: 1) el glucano (1,3- β y 1,6- β glucano), que se compone de 50 a 60% de la PCL, 2) la quitina, de 1 a 10% de la PCL e 3) la mananoproteína, de 30-40% de la PCL (Moreno *et al.* 2008).

La distribución de estos componentes está organizada en dos capas principales, la externa compuesta de mananoproteínas y cumple la función de reconocimiento, interacciones célula/célula e interacciones con el ambiente que determinan la especificidad inmunológica de la levadura. La capa interna, compuesta por β -glucanos y quitina, es responsable por la rigidez de la pared celular y define su forma (García, 2008; Gomes, 2009), siendo su estructura interconectada por ligaciones covalentes (Barata, 2012).

Las mananoproteínas poseen funciones enzimáticas y estructurales en la levadura, y también poseen una importante acción biológica en aves y porcinos, en nivel intestinal, aumentando la respuesta inmune, estimulando el crecimiento y mejorando la conversión alimenticia (Iglesias-Hernández *et al.* 2013). Las MP son polipéptidos altamente glicosilados que forman estructuras de grande porte en el exterior de la pared celular de la levadura (Lipke & Ovalle, 1998). Muchas mananoproteínas en la levadura presentan glicanos ligados al nitrógeno con una estructura de manosa y glicosaminoglicanos (Lipke & Ovalle, 1998). Las mananoproteínas son constituidas de 50 a 200 unidades de manosa, con ligaciones glicosídicas α -1,6, α -1,2 e α -1,3. (Lipke & Ovalle, 1998)

Estudios usando las fracciones de levaduras ricas en mananos presentan efectos benéficos sobre la salud y la productividad animal (Torrecillas *et al.*, 2013; Edwards *et al.*, 2014). Los prebióticos más utilizados ricos en manano son los mananoligosacáridos. La parte manano de este prebiótico actúa en las bacterias Gram-negativas por dos mecanismos: aglutinación de las fimbrias tipo I y modulación de la respuesta inmune del huésped, siendo el primero aparentemente el más discutido en la literatura (Costa, 2014). La colonización intestinal es reconocida como punto inicial de la infección bacteriana en los enterocitos; las lectinas manose-específicas de las fimbrias tipo I de las bacterias reconocen y se ligan a las glicoproteínas en las células del huésped (ricas en manose) (Moran, 2004). De esta forma, ocurre el bloqueo competitivo de las lectinas de las fimbrias bacterianas, impidiendo la infección bacteriana de los enterocitos (Moran, 2004).

Como la mananoproteína (Actigen[®]TM) es un producto derivado del mananoligosacárido, hace que la estructura del MOS responsable por la aglutinación de

bacterias sea suministrada de forma concentrada, aumentando así la probabilidad de la unión prebiótico-bacteria y minimizando las interacciones de bacterias Gram- negativas con los enterocitos (Hooge *et al.* 2013).

3.2. Uso de mananoproteína en la alimentación animal

Estudios que evalúan el uso de mananoproteína (MP) como aditivo alimentario en la alimentación animal son escasos y, hasta el presente, no fueron encontrados estudios en la literatura que evalúen su uso en la acuicultura.

Los pocos estudios publicados fueron realizados con pollos y porcinos. Donde la inclusión de 0,4 g kg⁻¹ de MP en la dieta y su asociación al antibiótico Halquinol no resultó en la mejoría de los parámetros zootécnicos de pollos (Barata, 2012). Sin embargo, la MP tuvo un efecto protector de la integridad intestinal de los pollos (Barata, 2012). Lo mismo fue observado por Moralez-López *et al.* (2009), donde las dietas de pollo de corte fueron suplementados con 95 mg kg⁻¹ hasta 190 mg kg⁻¹, presentando solamente aumento en las velocidades intestinales del tracto digestivo.

En estudios realizados con porcinos, se observó menor prevalencia de la *Salmonella* en la carcasa pre-sacrificio en porcinos alimentados con 1,6 g kg⁻¹ en el desmame (0-35 días), 0,8 g kg⁻¹ de MP en la dieta de 36 a 50 días e 0,4 g kg⁻¹ de 51 a 100 días (Costa, 2014). Adicionalmente, hubo aumento del crecimiento e incremento de la carcasa en porcino alimentado con 0,4 g kg⁻¹ (0 a 38 días) y 0,2 g kg⁻¹ (39 a 80 días) de MP en la dieta (Edwards *et al.* 2014).

1 Ácidos orgánicos y sus sales

Como alternativa adicional al uso de antibióticos, Vázquez *et al.* (2005) propusieron el uso de ácidos orgánicos como aditivos en la alimentación de peces, demostrando que bacterias lácticas inhibieron las bacterias patógenas de la microbiota de *Scophthalmus maximus* debido a la producción de ácido láctico y ácido acético sin la producción de bacteriocinas. Estos ácidos orgánicos han recibido gran atención como sustituyentes potenciales de los antibióticos con el fin de mejorar el rendimiento y la salud animal (Lückstädts 2008).

Los ácidos orgánicos se consideran compuestos GRAS (de inglés, Generally Regarded as Safe) con uno o más grupos carboxílicos (-COOH) con capacidad antimicrobiana (Deifoirdt *et al.* 2009). Muchos de los ácidos orgánicos también están disponibles en sales de sodio, potasio y calcio, lo que les confiere ventajas como ser inodoros, menos corrosivos, más solubles en agua que los ácidos libres y de fácil manipulación durante la adición en la ración debido a su solidez y poca volatilización (Partanen & Mroz 1999).

1.1 Mecanismos de acción y efecto de las sales orgánicas en la acuicultura

1.1.1 Inhibición de bacterias patógenas

Los ácidos orgánicos y sus sales son efectivos en la inhibición de las bacterias patógenas, principalmente gram negativas, al disminuir el pH de su entorno. Estos ácidos entran en la pared celular y liberan protones en el citoplasma desequilibrando el pH de la célula. Para mantener el equilibrio, la bacteria comienza a consumir grandes cantidades de ATP para expulsar los protones de la célula. El alto gasto de ATP causa una depleción energética que termina con la muerte celular (Lückstädts 2008).

Algunos ácidos orgánicos o sus sales ya han demostrado su poder inhibitorio *in vitro* frente a diferentes especies de *Vibrio*. Silva *et al.* (2013) verificaron que el fumarato, acetato, butirato y propionato de sodio fueron capaces de inhibir *V. alginolyticus*, *V. anguillarum* y *V. harveyi*. Estudios similares también demostraron la capacidad inhibitoria de ácidos orgánicos como el ácido fórmico, acético, butírico, propiónico y valérico frente a diferentes especies de *Vibrio* patógenos en crustáceos (Mine & Boopathy 2011; Adams & Boopathy 2013).

Además de disminuir el pH intracelular, los ácidos orgánicos y sus sales son capaces de formar complejos quelantes con minerales, principalmente con el hierro, indisponibilizando micronutrientes y limitando el crecimiento de otros microorganismos (Cardoso & Nogueira 2007).

1.1.2 Mejora en la digestión de nutrientes

Estudios han comprobado que el uso de ácidos orgánicos aumenta la absorción del fósforo en animales al disminuir el pH y consecuentemente solubilizando sustancias antinutricionales presentes en la ración, como fosfato tricálcico y fitatos. Además, los ácidos orgánicos son capaces de suministrar los minerales de las dietas bajando el pH, resultando en una mayor disociación de los compuestos minerales, reduciendo la tasa de vaciamiento del estómago y formando complejos minerales quelatos fácilmente absorbidos en el intestino. De igual forma pueden ser fuentes de energía que pueden ser usadas en varias rutas metabólicas energéticas como el ciclo de Krebs (Luckstadt 2008).

En el camarón marino *L. vannamei*, sales como el butirato y el propionato de sodio ayudaron a incrementar la retención de nitrógeno, la tasa de eficiencia proteica y la digestibilidad aparente de energía y fósforo (Silva *et al.* 2013; Silva *et al.* 2014), lo cual indica que el uso de ácidos orgánicos puede ayudar a incrementar la biodisponibilidad de nutrientes, mejorando la digestibilidad y consecuentemente disminuyendo residuos en los cultivos. Asimismo, el uso de ácidos orgánicos también puede influir en enzimas como la pepsina, la cual tiene mejor actividad a pH bajos (Luckstadt 2008). Según Silva *et al.* (2015) el uso de acetato y propionato de sodio incrementó la actividad *in vitro* de la tripsina y quimiotripsina en *L. vannamei* y el uso de fumarato y succinato de sodio aumentó la digestibilidad de proteína.

1.1.3 Mejora en los índices zootécnicos

Diversos estudios han demostrado que la inclusión de ácidos o sales orgánicas en dietas mejoran diversos parámetros zootécnicos en la acuicultura tales como: parámetros zootécnicos (ganancia de peso, eficiencia alimentaria, supervivencia), parámetros inmunológicos, microbiota intestinal y resistencia frente a desafío con diferentes patógenos.

En *L. vannamei* el uso de ácidos orgánicos o sus sales aumentaron la ganancia de peso, mejoraron la supervivencia y la eficiencia alimentaria y disminuyeron concentraciones de bacterias patógenas (*Vibrio* spp.) en el intestino (Silva *et al.* 2013, Silva *et al.* 2014). En *P. monodon* ácidos orgánicos además de disminuir las concentraciones de

Vibrios spp. en el hepatopancreas e intestino, confirieron mayor resistencia a los camarones frente a la infección con *V. harveyi*, aumentando la actividad inmune y reduciendo las lesiones en el hepatopancreas (Ng *et al.* 2015; Romano *et al.* 2015). Adicionalmente, en *L. vannamei* el uso del diformiato de potasio (KDF) aumentó el crecimiento y la productividad final en el 19.5% (Kühlmann *et al.* 2011a) y al mismo tiempo disminuyó la mortalidad en camarones infectados por *V. harveyi*, sugiriendo que este ácido también tendría el mismo efecto en otras bacterias gram negativas patógenas en el cultivo de *L. vannamei* (Kühlmann *et al.* 2011b).

4. Algas pardas

4.1. Producción y aplicación económica

Las plantas acuáticas son cultivadas en cerca de 50 países, en la última década, este sector económico se expandió en torno de 8% al año, más que duplicando la producción en este período. En 2014, la producción mundial ha alcanzado cerca de 28,5 millones de toneladas, gran parte dominada por algas pardas macroalgas, siendo el alga roja *Kappaphycus alvarezii* la especie más producida en el mundo, con producción aproximada de 11 millones de toneladas, seguida por el alga parda *Saccharina japonica*, con aproximadamente 7,7 millones de toneladas. Las algas pardas *Undaria pinnatifida* y *Sargassum fusiforme* están en el cuarto y sexto lugar entre las 10 especies más producidas en el mundo, con producción de 2,4 millones y 175 mil toneladas, respectivamente (FAO, 2016).

Este volumen producido de macroalgas fue destinado para el consumo humano, directo o de forma procesada (tradicionalmente en Japón, Corea y China), para el uso como fertilizante, biocombustible, para industria farmacéutica, cosmética, bien como para extracción de compuestos con acción gelificante (especialmente agar, carragenina y alginato) y de saborizantes para incorporar en comidas y bebidas industrializadas. Además de eso, por muchos años las macroalgas vienen siendo utilizadas para alimentación animal y en la medicina, como fuente de yodo, vermífugo y antibiótico natural. Actualmente, hay un creciente interés en dicha aplicación debido al valor nutricional y a la variedad de compuestos bioactivos de las macroalgas (FAO, 2016; Milledge *et al.* 2016). Similarmente, en la acuicultura diversos estudios han sido desarrollados evaluando las macroalgas como

ingredientes de dietas prácticas, generalmente en sustitución de la harina de pescado y/o a ingredientes de origen vegetal, o como aditivos alimentarios (en la forma íntegra y como extracto) con enfoque en los efectos funcionales sobre la fisiología y respuesta inmune de los animales (Costa *et al.* 2013; Cruz-Suárez *et al.* 2008; Niu *et al.* 2015; Schleder *et al.* in press).

4.2. Propiedades bioquímicas y nutraceuticas

Las algas pardas (Ochrophyta, Phaeophyceae) se destacan por sus diversas propiedades terapéuticas y abundancia de compuestos bioactivos, en especial los polisacáridos, fenoles y polifenoles, lípidos y los terpenoides (Balboa *et al.* 2013). Entre los polisacáridos, se destacan principalmente el fucoidan, el alginato y la laminarina. Esta última es compuesta por residuos de (1,3)- β -D-glucopiranososa, presenta un relativo bajo peso molecular y sus características estructurales varían entre las especies de algas pardas. Puede actuar como modulador del metabolismo intestinal, activador del sistema inmune, sin embargo presenta baja capacidad antioxidante. El alginato o ácido algínico es un polisacárido lineal aniónico que contiene residuos (1,4)- β -D-ácido manurónico y α -L-ácido gulurónico, comercialmente utilizado como agente gelificante (Balboa *et al.* 2013). El alginato de sodio estimula las defensas antioxidantes, la respuesta inmune y la resistencia contra infección bacteriana en camarones (Liu *et al.* 2006).

Los fucoidanos son polisacáridos sulfatados complejos encontrados en la matriz extracelular y pared celular de algas pardas. Representan del 10 al 20% de la masa seca de dichas algas y, de forma general, son compuestas por l-fucosa, grupos sulfatados y una o más porciones pequeñas con d-xilosa, d-manosa, d-galactosa, l-ramnosa, arabinosa, d-glucosa, d-ácido glicurónico y grupos acetil. Los fucoidanos son encontrados en algas pardas y en animales equinodermos, sin embargo no son encontradas en otros grupos de algas y tampoco en plantas terrestres. Fucoidanos provenientes de algas pardas presentan mayor complejidad y heterogeneidad en su estructura y composición que las sintetizadas en equinodermos. Existe una amplia cantidad de estudios relacionados con las actividades terapéuticas de los fucoidanos en diferentes seres vivos, demostrando actividades antitumorales, antivirales, antibacterianas, anti-inflamatorias, inmuno-moduladoras, de protección contra radiación, osteoartritis, úlceras gástricas, daños oxidativos e incluso

actuando como promotor de crecimiento y en la regeneración ósea (Balboa *et al.* 2013; Milledge *et al.* 2016). En camarones, el uso de fucoidanos extraídos de diferentes especies de algas pardas han demostrado resultados significativos, incrementando la respuesta inmune, resistencia a infección con virus de la Mancha Blanca (WSSV, del inglés *White Spot Syndrome Virus*) y *Vibrio* spp., e incluso en el incremento de peso (Chen *et al.* 2016, Immanuel *et al.* 2012, Sivagnanavelmurugan *et al.* 2014).

Los terpenos abarcan un gran número de metabolitos secundarios sintetizados con unidades de isopreno, el cual es responsable por la amplia diversidad estructural y funcional de estos compuestos. Basado en la cantidad de unidades de isopreno, los terpenos son clasificados como mono-, sesqui-, di-, sester-, tri- y tetraterpenos. Los esteroides están entre los triterpenos, y diferentes tipos de esos lípidos son encontrados en las algas pardas, tales como etilenocolesterol, colesterol, ergosterol y fucosterol, siendo este último el más predominante en este grupo de algas. El fucosterol posee diversos efectos benéficos, incluyendo inhibición de la absorción del colesterol, antitumorígeno, antioxidante, antidiabético, anti-inflamatorio, antibacteriano, antimicótico y antiulcerogénico. Además, otro grupo importante de terpenos son los carotenoides, los cuales son clasificados como tetraterpenos con ocho unidades de isopreno. En las algas pardas se puede destacar la fucoxantina, un carotenoide con diversas funciones terapéuticas, tales como protección contra rayos UV, anti-obesidad, anti-inflamatoria, antiangiogénico, antidiabético, antioxidante y antitumorígeno. La fucoxantina es encontrada en altas concentraciones en las algas *Undaria pinnatifida* y *Saccharina* sp., y es el principal producto extraído de la alga *Sargassum muticum* (Balboa *et al.* 2013; Fernando, Nah & Jeon, 2016; Milledge *et al.* 2016).

Los compuestos fenólicos representan un grupo amplio y diverso de metabolitos secundarios constituidos de uno o más grupos fenoles. Con base en sus propiedades estructurales fundamentales, los compuestos fenólicos varían de simple fenoles a moléculas complejas tales como ácidos fenólicos, flavonoides, florotaninos, coumarinas, ligninas, lignanas, estilbenos y sus derivados. Estos compuestos son reconocidos por sus extensas actividades bioactivas, incluyendo antioxidante, anti-inflamatoria, antitumorígena, antimicrobiana, entre otras. Las macroalgas marinas, especialmente las algas pardas, han sido extensamente estudiadas en lo que se refiere a su rico contenido de compuestos

fenólicos. En este grupo de macroalgas, se pueden destacar los florotaninos y el floroglucinol debido a su importancia como compuestos bioactivos (Fernando, Nah & Jeon, 2016).

Schleder *et al.* (in press) observaron que camarones *Litopenaeus vannamei* alimentados con una dieta con 4% de biomasa seca de *U. pinnatifida* presentaron mayor actividad de la enzima fenoloxidasas (PO), ligada al sistema inmunológico, y menor concentración de *Vibrio* spp. en el tracto digestivo. El análisis bioquímico de la biomasa seca de esa alga parda reveló un mayor contenido de compuestos fenólicos, flavonoides y carotenoides en relación a otra especie de alga parda evaluada (*Sargassum filipendula*), la cual no afectó el sistema inmunológico ni tampoco la microbiología del tracto digestivo de los camarones. En este sentido, Niu *et al.* (2015) también relataron aumento de la actividad de la PO en camarones *Penaeus monodon* alimentados con dietas con de 1 a 6% de biomasa seca de *U. pinnatifida*. Adicionalmente, camarones alimentados con dieta con 4% de biomasa seca de *U. pinnatifida* presentaron mayor resistencia a la infección con WSSV, con mortalidad 26% menor en relación al grupo control (Peruch *et al.*, 2016). Y además, las algas pardas *S. filipendula* y *U. pinnatifida* presentaron efecto sinérgico sobre la resistencia de los camarones al WSSV, pues las combinaciones 0,5%:2% y 0,5%:4% (*S. filipendula*:*U. pinnatifida*, respectivamente), redujeron en torno de 50% la mortalidad de los animales (Schleder *et al.* 2017). Estos datos refuerzan el efecto inmunomodulador y antimicrobiano de las algas pardas, en especial de *U. pinnatifida*, sobre camarones marinos.

Por otra parte, las algas pardas son ricas en otros compuestos, como ácidos grasos saturados y poli-insaturados, vitaminas, especialmente vitamina C y E, y sales minerales, principalmente selenio, zinc, manganeso y cobre. Estos compuestos están asociados a diversas propiedades, en especial antioxidante e anti-inflamatoria (Balboa *et al.* 2013; Fernando, Nah & Jeon, 2016). Por último, las propiedades nutricionales y funcionales de las macroalgas pueden incrementar el rendimiento zootécnico de camarones cultivados. Cruz-Suárez *et al.* (2008) relataron que la inclusión de 4 a 10% de algas del género *Sargassum* en la dieta incrementó significativamente el crecimiento del camarón blanco del Pacífico, y Niu *et al.* (2015) observaron que la adición de 1 a 3% de biomasa seca de *U. pinnatifida* incrementó el rendimiento zootécnico de *P. monodon*, no obstante los niveles de 5 y 6% redujeron el crecimiento de los animales. Camarones *L. vannamei* alimentados con

dietas con 0,5, 2 y 4% de las algas *S. filipendula* y *U. pinnatifida* presentaron rendimiento zootécnico similar al grupo control, sin embargo, todos los niveles de *S. filipendula* y el nivel de 4% *U. pinnatifida* incrementaron la actividad de la enzima digestiva amilasa, y el nivel 0,5% de ambas algas pardas provocaron el aumento de la superficie de absorción del intestino de los camarones después de 5 semanas de cultivo (Peruch *et al.* 2016).

4.3. Resistencia al estrés térmico

La aparición de enfermedades en la acuicultura, así como en cualquier sector de producción animal, procede de la interacción entre huésped, ambiente y patógeno. De esta forma, condiciones ambientales adversas, como mala calidad del agua, exceso de manejo de los animales, alta densidad y variaciones extremas o bruscas de temperatura constituyen importantes gatillos para surtos de enfermedades, una vez que perjudican el rendimiento zootécnico y la respuesta inmunológica de los animales, tornándolos más susceptibles a los agentes patogénicos (Kautsky *et al.* 2000; Reverter *et al.* 2014).

Las variaciones de temperatura son particularmente perjudiciales para los organismos ectotérmicos, tales como los camarones, una vez que dichos animales no pueden controlar temperatura interna del organismo. En este sentido, animales ectotérmicos cuando son expuestos al frío demuestran diversos cambios fisiológicos, entre los cuales se destacan los desórdenes neuromusculares causados por la reducción del gradiente de iones y disfunción de las sinapsis; pérdida de la fluidez de la membrana y mal funcionamiento de las proteínas transmembranas; degradación y agregación de las proteínas; alteraciones en la síntesis de metabolitos y en el metabolismo energético (Hayward, Manso & Cossins, 2014).

Por consiguiente, animales de regiones templadas (animales tolerantes al frío) desarrollaron mecanismos bioquímicos y fisiológicos para superar el estrés causado por el frío. Para los insectos, fueron descritos algunos mecanismos relacionados a la capacidad de resistir a esta condición, tales como aumento de la expresión de las acuaporinas, de las enzimas de detoxificación y de las proteínas de choque térmico, las cuales son responsables, respectivamente, por facilitar el movimiento del agua entre los compartimentos celulares, reducir el estrés oxidativo e inhibir la desnaturalización proteica. Esos insectos también son capaces de incrementar la síntesis y/o almacenamiento de metabolitos, tales como aminoácidos (ej.: prolina y arginina), carbohidratos (ej.: glucosa y

trehalosa) y polalcohol (ej.: glicerol, sorbitol e inositol), los cuales actúan como potentes crioprotectores (Hayward, Manso & Cossins, 2014; Teets & Denlinger, 2013). Estas moléculas son ampliamente conocidas como osmólitos orgánicos, cuyas funciones comprenden estabilizar las biomembranas y proteínas, bien cómo prevenir el daño osmótico en las células (Yancey, 2005).

Además, insectos que presentan resistencia al frío normalmente modifican su metabolismo energético, inhibiendo la vía aeróbica y estimulando la glucólisis. Ellos también reestructuran los mecanismos de transporte de iones, que son usualmente perjudicados debido a la reducción de la fluidez de la membrana y de la síntesis de ATP (Teets & Denlinger, 2013). La reducción de la fluidez de la membrana durante el enfriamiento es particularmente perjudicial para el funcionamiento celular, pues causa la inmovilización de las proteínas transmembranas, perjudiciales en el proceso de señalización celular y transporte de nutrientes/iones. La adaptación de la homeoviscosidad de la membrana es un importante mecanismo para resistir al frío (Hayward, Manso & Cossins, 2014).

Por otro lado, animales intolerantes al frío pueden tornarse resistentes a través de la suplementación alimentaria. En peces, Harpaz, Becker y Blum (1999) observaron el aumento de por lo menos 50% en la sobrevivencia de *Pelvicachromis pulcher* alimentado con diferentes niveles de L-carnitina, después de 24 h de choque térmico. Además, el camarón (*L. vannamei*) cuando alimentado con dietas con 0,5% y 2% de la alga parda *S. filipendula* demostraron sobrevivencia significativamente mayor (97% en ambos tratamientos) en relación a los camarones alimentados con dietas sin suplementación (43%) después del choque térmico, ya la adicción de 4% de esa misma alga no afectó en la resistencia de los animales. Por otro lado, los crecientes niveles (0,5, 2 e 4%) de la alga parda *U. pinnatifida* causaron aumentos progresivos en la mortalidad de los camarones después del choque térmico, llegando a 100% en los animales alimentados con la dieta con el mayor nivel de adición (Schleder *et al.* in press).

A través de la utilización de la técnica de espectrometría de masas MALDI-TOF (de sus siglas en inglés *Matrix-Assisted Laser Desorption/Ionization - Time-Of-Flight*), fueron analizados los perfiles de proteínas y lípidos de los hemocitos de camarones alimentados con 0,5% de *S. filipendula*, antes y 15 minutos después del choque térmico, con el objetivo

de comprender los posibles mecanismos fisiológicos asociados con la resistencia observada. Se identificó que la resistencia podría estar asociada al aumento de la fluidez de la membrana y de la defensa antimicrobiana, a la reducción del estrés oxidativo y de la muerte celular causada por estrés, y a la modulación diferencial del metabolismo energético y de la regulación del ADN (Schleder *et al.* 2016). Además de eso, la combinación de ambas especies de algas pardas en la dieta (0,5%:1%, 0,5%:2% y 0,5%:4% de *S. filipendula* y *U. pinnatifida*, respectivamente) demostró un efecto sinérgico sobre la resistencia de los camarones al choque térmico, una vez que los animales alimentados con las dietas con las combinaciones de las algas presentaron una tasa de mortalidad similar a los animales del grupo control (sin adición), de esta forma la adición de 0,5% de *S. filipendula* fue capaz de evitar el efecto negativo de la adición de diferentes niveles de *U. pinnatifida*. Sin embargo, los autores sugirieron que futuros estudios deben ser realizados con mayores niveles de *S. filipendula* para evaluar si causaría reducción en la mortalidad después del choque térmico (Schleder *et al.* 2017).

En este contexto, la utilización de la biomasa seca de algas pardas como aditivo alimentario para camarones marinos presenta un enorme potencial, pues además de causar el aumento en la resistencia de los animales al estrés térmico, provocaron la modulación del sistema inmunológico de los mismos, tornándolos más resistentes a las enfermedades y a sus mecanismos desencadenadores, tales como la variación de temperatura. Este hecho presenta mayor relevancia cuando se considera la creciente expansión de la producción mundial de las macroalgas, y el bajo valor agregado para producción de la biomasa seca y, en consecuencia, para inclusión en las dietas comerciales. Además de eso, actualmente hay una tendencia creciente de utilizar productos naturales para alimentación animal, como sustituto a los antibióticos y otros fármacos, el uso de biomasa algácea un enfoque sostenible y ambientalmente amigable, pues tiene como característica ser biodegradables, manteniendo la calidad del agua dentro de los estándares de seguridad sanitaria y ambiental.

5. Conclusión

El uso de aditivos alimentarios no es una solución única contra las enfermedades en la camaronicultura marina, sin embargo pueden hacer parte de un componente de

estrategias que en conjunto pueden incrementar los índices productivos, y auxiliar a enfrentar las enfermedades.

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La Nutrición y Alimentación del Pepino de Mar (Echinodermata, Holothuroidea); Situación Actual y Perspectivas para el Desarrollo de su Cultivo Intensivo

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Resumen

La elevada demanda de pepino de mar en el sureste asiático y particularmente en china, ha generado una sobreexplotación de existencias silvestres a escala mundial, provocando el agotamiento de pesquerías y desaparición de poblaciones, por lo que su cultivo se aprecia como una alternativa para satisfacer la demanda. Su cultivo se inició a mediados del siglo XX en China, donde actualmente la oferta local proviene exclusivamente de cultivos extensivos y semiintensivos en estanques costeros, encierros en el mar, así como “sea ranching”. El cultivo intensivo está poco desarrollado, atribuido a la carencia de alimentos apropiados para las diferentes etapas de desarrollo del organismo, asociados al bajo conocimiento de requerimientos nutricionales e información para su formulación. Este organismo es bentófago detritívoro, consumiendo materia orgánica en degradación con restos animales y vegetales acompañados de microorganismos epibentónicos. Sus requerimientos proteicos y lipídicos rondan en 20 y 2-3% de la dieta, respectivamente. Existe información sobre sus necesidades de vitaminas C y E, así como de uso ingredientes, que indica que sus dietas se pueden formular a base de harinas de soya, macroalgas y subproductos agroindustriales. Se detectan necesidades de información necesaria para la elaboración de dietas apropiadas para su cultivo intensivo.

Palabras clave: Holoturidos; Acuicultura; Algas; Detritus

Abstract

The high demand for sea cucumbers in Southeast Asia, and particularly in China, has led to the overexploitation of wild stocks worldwide, leading to the depletion of fisheries and the loss of entire populations, so their cultivation is considered as an alternative to satisfy the market. Its cultivation began in the middle of the 20th century in China, where the local supply now comes exclusively from extensive and semi-intensive farming in coastal ponds, sea enclosures, and sea ranching. Intensive farming is poorly developed, attributed to the lack of appropriate feeds for the different developmental stages of the organism, associated to the low knowledge of nutritional requirements and information for their formulation. This benthic organism ingests detritus, consuming organic matter in degradation with animal and vegetable remains, accompanied by epibenthic microorganisms. Their protein and lipid requirements are around 20 and 2-3% of the diet, respectively. There is information about their needs for vitamins C and E, as well as the use of ingredients, which indicates that their diets can be formulated from soybean and macro-algae meals, and agro-industrial by-products. The review identifies information needs, necessary for the development of appropriate feeds for intensive farming.

Keywords: Holoturid, Aquaculture, Algae, Detritus

1. Introducción

La pesquería de pepino de mar (Echinodermata, Holothuroidea) es una actividad relativamente reciente en México, ya que se inició en la península de Baja California a finales de la década de los 80's (INAPESCA 2012), incidiendo sobre dos especies, *Isostichopus fuscus* (Ludwig) y *Parastichopus parvimensis* (Clark). La intensidad de la captura tuvo un efecto severo sobre la sustentabilidad de las poblaciones, lo que obligó al cierre de la pesquería a mediados de los 1990's, por lo que hasta la fecha *I. fuscus* se considera como especie en riesgo sujeta a protección especial (IUCN 2017-1). Ante la suspensión de la pesquería de este recurso en el océano Pacífico, la actividad se trasladó al Golfo de México, donde desde inicios de los años 2000 se enfoca fundamentalmente a la captura de *Isostichopus badionotus* (Selenka), especie distribuida en el Atlántico occidental, desde el sur de Estados Unidos hasta el norte de Brasil incluyendo el Mar Caribe, así como la isla Ascensión en el Atlántico central y el Golfo de Guinea en África occidental (IUCN, 2017-1), siendo objeto de captura regulada o ilegal en la mayoría de los países donde está presente, para ser exportada en forma seco-salada como “bêche-de-mer” o “trepan” al mercado asiático donde es altamente demandada.

A partir del 2010 la producción pesquera de pepino de mar se concentra en la Península de Yucatán, lo que se refleja en un incremento sustancial en la producción de este recurso (Conapesca 2017). Como se observa en la Figura 1, hasta antes de la pesquería de *I. badionotus* se registraba una producción promedio de pepino de mar de alrededor de 450 t por año, volumen que se incrementa hasta las 2,600 t en el 2010 cuando inicia la pesca comercial en Yucatán, con una reducción gradual hasta el 2012 y un nuevo pico de 2,760 t en el 2013, momento en que nuevamente se presenta un marcado declive en la producción, explicado por un incremento sostenido en el esfuerzo pesquero tanto en la época autorizada de captura, como por su captura ilegal durante todo el año en época de veda.

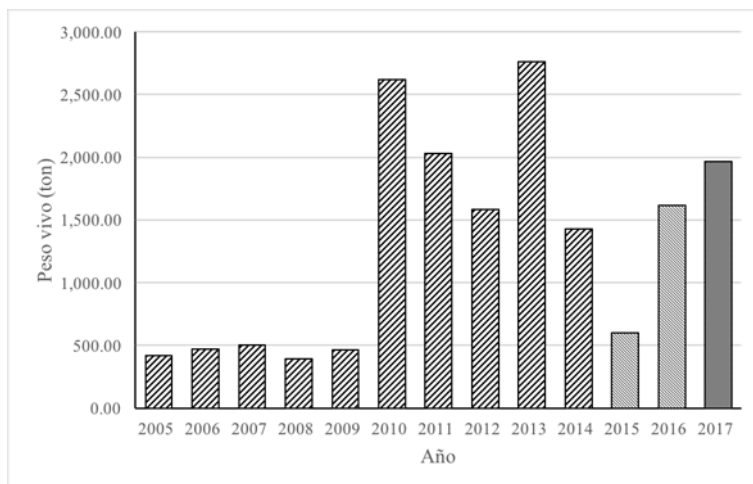


Figura 1. Producción de pepino de mar en México. Los valores de 2005 a 2014 corresponden a estadísticas oficiales (Conapesca 2017); los de 2015 y 2016 se obtuvieron a partir de declaraciones de la Conapesca publicadas en diarios locales, y la del 2017 representa la cuota autorizada para capturar del 30 de mayo al 14 de junio.

El pepino de mar es un organismo bentónico altamente vulnerable a la pesca debido a sus hábitos de vida y reproductivos, ya que posee una movilidad muy limitada, carece de estructuras o medios de defensa, y forma agregaciones reproductivas sobre las que se ejerce directamente la pesca mediante buceo autónomo, por lo que es muy fácil agotarlas.

Estos organismos son capturados en zonas costeras y arrecifales de aguas someras en la mayoría de los países tropicales, donde después de ser cocidos, salados y secados, son enviados al mercado asiático, especialmente China, donde alcanzan precios muy altos por su elevada demanda, lo que ha estimulado la sobreexplotación que ha resultado incluso en la desaparición de poblaciones alrededor de mundo. Se considera, por ejemplo, que a nivel global se explotan alrededor de 66 especies, de las cuales el 20% están agotadas, 38% sobreexplotadas, 15% explotadas al límite de su capacidad y 27% subexplotadas (Fabinyi & Liu 2014; Poot-Salazar, Hernández-Flores & Ardisson 2015). Esta situación ha dado lugar a la aplicación de medidas de regulación a la captura y en algunos casos el cierre de pesquerías comerciales, con efectos adversos sobre la economía y bienestar de los pescadores que dependen de este organismo para su subsistencia (Purcell, Polidoro, Hamel, Gamboa & Mercier 2014; Purcell, Ngaluafe, Foale, Cocks, Cullis & Lalavanua 2016).

Debido a la vulnerabilidad de las poblaciones de pepino de mar y el agotamiento de sus pesquerías, se considera que la acuicultura es la alternativa más viable para satisfacer la creciente demanda de “bêche de mer” (Purcell, Hair & Mills 2012; Han, Keesing & Liu 2016), además de ser una opción productiva que además de contribuir a su producción ordenada, permitiría reducir la presión pesquera sobre las poblaciones silvestres y apoyar acciones para su repoblamiento y restauración.

La nutrición y alimentación es uno de los factores que más influyen sobre el éxito de un cultivo, por lo que en este documento se discute el estado actual del conocimiento que sobre este tema se tiene de las especies de pepino de mar sujetas a cultivo comercial.

1.1. Situación actual de su cultivo

Las acciones para el cultivo de este organismo se iniciaron en China en 1954 con la reproducción en cautiverio de *Apostichopus (Stichopus) japonicus* (Selenka), especie de aguas templadas y frías, principalmente explotada en el SE asiático (Liu, Sun & Liu 2015), mientras que en aguas tropicales los esfuerzos se han orientado fundamentalmente al cultivo de *Holothuria scabra* Jaeger en la región del Indo Pacífico (Purcell *et al.* 2012). De acuerdo con las estadísticas de la FAO (2017), en el 2014 la producción de pepino de mar por acuicultura alcanzó 201,069 t, las cuales fueron obtenidas principalmente en China, donde desde el año 2000 la totalidad de la producción proviene exclusivamente de granjas acuícolas (Han *et al.* 2016).

Los métodos de cultivo aplicados son muy diversos, condicionados por la capacidad técnica de los acuicultores y la rentabilidad económica. La mayor parte de la actividad se realiza a escala extensiva o semi-intensiva en estanques de camarón abandonados o estanques de marea construidos ex profeso, así como en encierros en el mar o mediante la liberación de juveniles en áreas controladas y repoblación de zonas agotadas (Purcell *et al.* 2012; Han *et al.* 2016). Estos métodos son los más populares en China y países del Indo Pacífico, especialmente porque no requieren de suministro de alimento ya que los organismos utilizan la productividad natural en los sistemas. Los cultivos en estanques pueden ser monoespecíficos o en policultivo con camarones o peces, donde aprovechan el alimento no consumido y los desechos de los animales. Un modelo de cultivo que ha cobrado auge en

los últimos tiempos es el de los cultivos integrados multitróficos, donde se utiliza el pepino de mar como especie limpiadora del fondo en granjas de moluscos bivalvos e incluso dentro de jaulas de peces (Chopin, Cooper, Reid, Cross & Moore 2012; Purcell *et al.* 2012; Granada, Sousa, Lopes & Lemos 2016; Han *et al.* 2016).

El cultivo de este organismo en México es reciente, realizándose de manera piloto comercial desde hace varios años en el Pacífico con base en *I. fuscus*, mientras que a partir del 2007 se realizan investigaciones en Yucatán enfocadas al desarrollo tecnológico para el cultivo de *I. badionotus*. En el año 2010 se obtuvieron en el Cinvestav-Mérida los primeros desoves que se tengan registrados para esta especie en condiciones controladas con fines acuaculturales, y a la fecha se han desarrollado protocolos de cría larvaria (Zacarías-Soto, Olvera-Novoa, Pensamiento-Villarauz & Sánchez-Tapia 2013), así como manejo y alimentación de juveniles (Martínez-Milián & Olvera-Novoa 2016) y adultos (Zacarías-Soto & Olvera-Novoa 2015).

2. Alimentación

Existen más de 1,400 especies y 160 géneros de pepino de mar, que se distribuyen desde la línea de marea hasta las profundidades abisales (Vergara & Rodríguez 2015; Zhao 2015). Las especies comerciales de pepino de mar pertenecen principalmente al orden Aspidochirotida, caracterizadas por ser organismos que viven asociados al fondo en zonas arenosas, de pastizales y arrecifales, donde se alimentan de detritus orgánicos, bacterias y diatomeas, junto con sedimento y fragmentos de plantas, entre otros materiales (Purcell, Lovatelli, Vasconcellos & Ye 2010; Xu, Hamel & Mercier 2015). Al respecto, Sambrano, Díaz & Conde (1990), Guzmán, Guevara & Hernández (2003) así como Vergara & Rodríguez (2015), señalan que *I. badionotus* vive preferentemente en aguas someras protegidas del oleaje, con fondos arenosos o fangosos, con parches de coral y de pastos marinos, con preferencia por estos últimos. En la plataforma costera de Yucatán se localiza además en suelos pedregosos, en profundidades superiores a los 10 m.

Sambrano *et al.* (1990) así como Vergara & Rodríguez (2015) al analizar el contenido intestinal de esta especie, señalan que su ingesta se compone de restos de macroalgas y de fanerógamas marinas, microalgas, foraminíferos, microcrustáceos y moluscos, junto con

Olvera-Novoa, M. et al., 2017. La Nutrición y Alimentación del Pepino de Mar (Echinodermata, Holothuroidea); Situación Actual y Perspectivas para el Desarrollo de su Cultivo Intensivo. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. . (Eds), Investigación y Desarrollo en Nutrición Acuícola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 106-155.

sedimento fino y otros restos orgánicos, con una preferencia por partículas de alimento y sedimento de entre 1.28 y 2.04 mm. No son selectivos en su tipo de alimento, por lo que la presencia de microcrustáceos o moluscos de diferentes tipos se atribuye a una ingesta accidental, más que a una acción deliberada, condicionada únicamente por el tamaño de partícula.

El cultivo intensivo de cualquier organismo acuático depende de la disponibilidad de alimentos formulados que satisfagan sus necesidades nutricionales, y que además sean aceptados e ingeridos por el animal. Dado que la mayor parte del crecimiento y engorda de este organismo con fines comerciales se realiza en sistemas semi-intensivos y extensivos mediante la liberación de juveniles al medio silvestre para posterior recaptura de adultos, en este tipo de cultivos su dieta depende de la disponibilidad de alimento natural, y generalmente no son utilizados alimentos completos o suplementarios, mientras que en el criadero se presenta una situación opuesta, ya que en estos se presenta la mayor demanda de alimentos naturales y artificiales de diversos tipos, para utilizarse durante la larvicultura y producción de juveniles, etapa en la que se requieren los mayores esfuerzos de investigación para identificar los alimentos más apropiados tanto para reproductores como para larvas y juveniles (Purcell *et al.* 2012).

El principal obstáculo para la elaboración de alimentos formulados para pepino de mar se relaciona con sus hábitos detritívoros, ya que se requiere de dietas completas microparticuladas que sean palatables, debido a que el animal selecciona su alimento por tamaño de las partículas que serán ingeridas utilizando sus palpos orales, siendo un reto el desarrollo de formulaciones que sean aceptadas por estos organismos.

Esta situación influye en la viabilidad para realizar estudios para la determinación de sus requerimientos nutricionales, lo que se traduce en lagunas del conocimiento relacionado con formulaciones para las diferentes etapas de crecimiento, tamaños de partícula apropiados, uso de aditivos y atractantes, entre otros aspectos, sin embargo, en los últimos años se ha liberado información generada especialmente en China, que contribuye en parte a resolver estas dudas.

2.1. Requerimientos nutricionales

El cultivo del pepino de mar en estanques está limitado por la falta de alimentos específicamente formulados, lo que se atribuye en parte a la falta de información sobre sus requerimientos nutricionales (Seo & Lee 2011). Sin embargo, en China se han realizado estudios para determinar los requerimientos de los principales nutrientes para *A. japonicus*. En la Tabla 1 se presentan resultados relevantes de estos esfuerzos.

Las investigaciones para determinar requerimientos de proteína indican que el pepino de mar necesita entre 11.8 y 20% de proteína, lo cual se asocia a su bajo nivel trófico y el pobre contenido de este nutriente en los sedimentos que ingiere. El nivel de proteína en la dieta también es afectado por su origen y los niveles de lípidos en el alimento. Al respecto, Seo & Lee (2011) observaron un mejor crecimiento cuando se alimenta a los juveniles con dietas a base de harina de soya con niveles de 20% de proteína y 2% de lípidos. Consideran que niveles elevados de lípidos afectan su tasa de crecimiento, posiblemente por acelerar el tiempo de evacuación intestinal que afecta la digestibilidad y asimilación de nutrientes, sin embargo, el nivel de proteína puede ser afectado por la presencia de antinutrientes y deficiencia de aminoácidos en la soya. Al respecto Bai, Zhang, Xia, Liu, Ru, Xu, Zhang & Yang (2016), confirman esta apreciación al evaluar el requerimiento de proteína de tres variedades de *A. japonicus* (verde, blanco y púrpura), estimando que el nivel óptimo para máximo crecimiento es de alrededor del 11%, un nivel menor que explican por haber usado una mezcla 1:1 de harina de pescado y de soya como fuente proteica. Por su parte, Liao, Ren, He, Jiang & Han (2014) utilizando una mezcla de harina de pescado y proteína de soya estimaron que el nivel óptimo de proteína para un máximo crecimiento se encuentra entre 10.8 y 13.5 %, observando que un exceso de proteína reduce la ingesta de alimento, lo que confirma que este organismo no requiere niveles elevados de este nutriente, cuyos niveles para un óptimo crecimiento deberían de estar en el intervalo que señalan (Tabla 1).

Tabla 1. Requerimientos nutricionales estimados para *A. japonicus*

Estadio de desarrollo	Nutriente	Ingredientes	Tipo	Nivel óptimo (%)	Referencias
Juvenil (1.3 g)	Proteína y lípidos	Harina de soya, <i>Sargassum</i> , aceites de hígado de calamar y de soya, premezclas de vitaminas y minerales	Polvo ($\approx 70 \mu$)	20% P + 2% L	Seo & Lee (2011)
Juvenil (1.05 g)	Proteína	<i>Sargassum</i> , levadura, proteína de soya, harina de pescado, premezclas de vitaminas y de minerales	Pélet (1.8 mm \emptyset)	10.8-13.5	Liao <i>et al.</i> (2014)
Juvenil (5 g)	Proteína	<i>Ulva lactuca</i> , <i>Laminaria japonica</i> , harina de pescado harina de soya, aceite de pescado, premezclas de vitaminas y minerales, polvo de concha de ostión, lodo marino	Polvo ($< 75 \mu$)	11	Bai <i>et al.</i> (2016)
Juvenil (0.65 g)	Lípidos	Harinas desengrasadas de <i>Sargassum</i> , pescado y soya; levadura, lodo marino, aceite de calamar, premezclas de minerales y vitaminas	Pélet (1.8 mm \emptyset)	1.9	Liao <i>et al.</i> (2017)
Juvenil (1.97 g)	HUFAs	Harinas desengrasadas de <i>Sargassum</i> y calamar, proteína de soya; levadura, lodo marino, aceite de hígado de calamar, premezclas de minerales y vitaminas	Pélet (1.8 mm \emptyset)	0.22-0.46	Liao <i>et al.</i> (2015)

Estadio de desarrollo	Nutriente	Ingredientes	Tipo	Nivel optimo (%)	Referencias
Juvenil (≈ 5 g)	Carbohidratos	Harina de pescado y soya, alga pulverizada, almidon gelatinizado de maíz, almidón de trigo, aceite de hígado de calamar, lecitina, premezclas de vitaminas y minerales, lodo marino	Pélet ($\approx 70 \mu$)	48.6-49.3	Xia <i>et al.</i> (2015)
Juvenil (1.48 g)	α -tocoferol	Caseina, harina de pescado desengrasada, harina de trigo, aceite de maíz, premezcla de vitaminas y de minerales	Pélet (180 μ)	23.1-41 mg kg ⁻¹	Ko <i>et al.</i> (2009)
Juvenil (7.96 g)	Vitamina E	Harinas de pescado y de soya, polvo de algas, lodo marino, polvo de conchas, almidón de trigo, premezclas de vitaminas y de minerales	Granulado (250 μ)	88-92 mg kg ⁻¹	Wang <i>et al.</i> (2013)
Juvenil (1.49 g)	Ácido ascórbico	Harina de pescado desengrasada, caseina libre de vitaminas, harina de trigo, premezclas de vitaminas y minerales	Granulado (180 μ)	105.3 mg kg ⁻¹	Okorie <i>et al.</i> (2008)
Juvenil (10.04 g)	Ácido ascórbico	Harinas de pescado y de soya, <i>Sargassum</i> , levadura, lodo marino, premezclas de vitaminas y minerales	Pélet (1.8 mm \emptyset)	598-1,473 mg kg ⁻¹ óptimo crecimiento 4,676 mg kg ⁻¹ mejor calidad carne	Ren <i>et al.</i> (2016)

Por otra parte, Liao, Ren, Chen, Han, Liu, Jiang & Wang (2017) estimaron que el nivel mínimo de lípidos para un máximo crecimiento de *A. japonicus* se encuentra en 1.9% en dietas con 14% de proteína. Este resultado confirma lo observado por Seo & Lee (2011), quienes obtuvieron el mejor crecimiento con 2% de lípidos. Liao *et al.* (2017) registraron además una reducción en la tasa de crecimiento al incrementar el contenido de lípidos en la dieta, en tanto que la actividad de lipasas intestinales aumentó al haber mayor contenido de lípidos dietarios. Igualmente confirmaron un incremento en el contenido de lípidos en el cuerpo con el aumento de su ingesta. Ellos opinan que el bajo requerimiento lipídico del pepino de mar es un reflejo de sus hábitos alimenticios, dado que los detritus orgánicos que consumen son pobres en este nutriente, y consideran que dependen en mayor medida de carbohidratos como fuente de energía.

Al igual que en el requerimiento de proteínas representa al de aminoácidos, el de lípidos se relaciona con su composición de ácidos grasos esenciales. Solamente Liao, Ren, Chen, Jiang, Yang & Han (2015) han abordado este tema con juveniles de *A. japonicus*. Ellos estudiaron el efecto de diversos niveles de ácidos grasos altamente insaturados (HUFA) en dietas con 13.5% de proteína y 1.7% de lípidos, sobre el desempeño de este organismo, observando un efecto positivo en el crecimiento con una concentración óptima de entre 0.22 y 0.44% de HUFAs en la dieta, y consideran también que no requieren niveles elevados debido a sus hábitos alimenticios donde este nutriente puede ser escaso, recomendando precaución al incrementar el contenido de ácidos grasos arriba de los niveles mencionados.

Se considera que los animales no tienen un requerimiento específico de carbohidratos, sin embargo y debido a su amplia disponibilidad y bajo costo, se utilizan hasta un cierto nivel como fuente de energía alternativa a proteínas y lípidos en la dieta, tomando en cuenta la tolerancia de los organismos hacia este nutriente que se asocia a sus hábitos alimenticios, por lo que su exceso provoca problemas metabólicos. Xia, Gao, Wang, Li, Zhang & Zhang (2015a) evaluaron el efecto de dietas con diferentes niveles de inclusión de carbohidratos para juveniles de *A. japonicus*, determinando que el pepino de mar es un organismo que crece y utiliza el alimento adecuadamente cuando recibe entre 48.6 y 49.3% de carbohidratos digeribles en su dieta, demostrando una elevada capacidad para utilizar este

ingrediente como fuente de energía, aspecto también considerado por Liao *et al.* (2017), posiblemente asociado a una ingesta elevada en su alimento natural con residuos de materia vegetal.

El estudio de requerimientos de vitaminas en pepino de mar es limitado, habiéndose demostrado únicamente la necesidad de incluir vitaminas C y E en su dieta para un adecuado desempeño. Ko, Go, Okorie, Kim, Lee, Yoo & Bai (2009) estudiaron el requerimiento de α -tocoferol en juveniles de *A. japonicus*, determinando que el nivel óptimo de este nutriente en base al peso ganado es de 23.1 mg kg⁻¹ de dieta, nivel que se incrementa hasta 44 mg kg⁻¹ para una máxima supervivencia. Ellos consideran que el α -tocoferol es esencial para este organismo, sin embargo, sus requerimientos son inferiores a los de peces y camarones, posiblemente debido al bajo nivel de lípidos presente en su dieta.

En un estudio más reciente, Wang, Xu, Li, Li, Bao, Che, Li & Jin (2015) evaluaron el requerimiento de vitamina E en juveniles de la misma especie, demostrando que este organismo necesita esta vitamina en su dieta para un adecuado crecimiento y respuesta de su sistema inmune, ubicando el requerimiento en 88-92 mg kg⁻¹ de dieta, nivel mayor al determinado por Ko *et al.* (2009) para α -tocoferol, y semejante al observado en diversas especies de peces. La diferencia en el requerimiento la explican en el hecho de que los juveniles de este estudio eran más grandes (7.96 g) que los utilizados por Ko *et al.* (2009) que solamente pesaban 1.48 g, lo que no aclara la diferencia ya que se esperaría un mayor requerimiento en juveniles tempranos con una tasa de crecimiento más activa y por lo mismo en este segundo experimento los niveles deberían de ser menores a los observados por Ko *et al.* (2009). La otra explicación que se da es que usaron una dieta práctica en lugar de la dieta semipurificada empleada por Ko *et al.* (*op. cit.*), y por lo mismo parte de la vitamina E se pudo haber utilizado como antioxidante en la dieta a base de harina de pescado y soya usada por Wang *et al.* (2013).

El ácido ascórbico (vitamina C) es esencial en la mayoría de peces e invertebrados acuáticos dada su incapacidad de sintetizarlo *de novo*, por lo que debe de estar presente en su dieta en cantidades suficientes para un adecuado crecimiento, respuesta al estrés, respuesta inmune, reparación de tejidos, procesos reproductivos y síntesis de colágeno

(Okorie, Ko, Go, Lee, Bae, Han & Bai 2008; Ren, Liao, Han, Li, Jiang & Wang 2016). En el caso del pepino de mar esta vitamina es particularmente importante, ya que actúa como cofactor en la hidroxilación de la prolina y lisina para la síntesis de colágeno, mismo que es el principal componente de la pared corporal de este organismo (Ren *et al.* 2016), por lo que su importancia en su metabolismo debe de ser de relevancia.

A pesar de su importancia, pocos son los estudios realizados para determinar su requerimiento, cada uno de los cuales muestran resultados diferentes. Okorie *et al.* (2008) estimaron que *A. japonicus* requiere entre 100 y 105.3 mg de vitamina C por kg de dieta para adecuado crecimiento, mientras que Ren *et al.* (2016), más recientemente consideran que los juveniles requieren entre 598 y 1,473 mg kg⁻¹ de ácido ascórbico para un óptimo crecimiento, nivel que sube hasta 4,676 mg kg⁻¹ para una buena textura de la carne y una adecuada condición en su sistema antioxidante.

Las discrepancias en la estimación de los niveles óptimos de micronutrientes, resaltan las dificultades para determinar los requerimientos en este organismo por sus hábitos alimenticios detritívoros, en cuya dieta natural se incluye una gran variedad de microorganismos como bacterias, hongos y microalgas, que le proporcionan vitaminas en cantidades apropiadas para satisfacer sus necesidades.

De acuerdo con estos resultados, está claro que este organismo dado su nivel trófico, donde consume materia orgánica en degradación, en conjunto con microorganismos del epibentos, posee bajos requerimientos nutricionales, con niveles de proteína de alrededor de 20% y de 2-3% de lípidos, además de bajas necesidades de vitaminas que son abundantes en los microorganismos de su dieta natural, sin embargo se nota una ausencia de información sobre sus necesidades nutrimentales en las fases de crecimiento y engorda así como para un adecuado mantenimiento de reproductores, siendo esencial estos estudios para contar con alimentos formulados para su cultivo en sistemas intensivos.

2.2. Ingredientes y alimentos

Uno de los retos para el cultivo del pepino de mar, primero en la etapa de criadero y de crecimiento, y eventualmente en sistemas intensivos en tanques, es el contar con dietas

completas que sean aceptadas y estimulen un adecuado crecimiento y salud de los organismos. La mayor parte de las acciones de alimentación artificial se basan en el uso de dietas naturales, en particular harinas de macroalgas, lo cual se ha convertido en un cuello de botella debido a su costo derivado de su sobreexplotación, en particular de *Sargassum thunbergii*, la especie de alga más utilizada para alimentación de *A. japonicus*.

Para resolver esta deficiencia y estar en capacidad de formular dietas prácticas y completas, se han realizado diversos estudios tanto con *A. japonicus* como con *H. scabra* e *I. badionotus* (Tabla 2), para identificar ingredientes e insumos que sean adecuadamente utilizados por los organismos, indicando que el pepino de mar, por sus hábitos alimenticios detritívoros con tendencia a la omnívoros y herbívoros, no necesita de ingredientes de alto costo, en particular harina de pescado, creciendo apropiadamente a base harinas de algas o con alimentos preparados con diversos subproductos agroindustriales, así como alimentos comerciales desarrollados para la larvicultura de peces y crustáceos (Giraspy & Ivy 2008; Liu, Dong, Tian, Wang & Gao 2010a; Seo, Shin & Lee 2011a,b; Martínez-Milián *et al.* 2016).

El uso de alimentos o suplementos para larvicultura ha resultado adecuado para la primera alimentación de juveniles tempranos, además de ser un buen suplemento de ácidos grasos para el mantenimiento de reproductores. Giraspy & Ivy (2008) estudiaron el impacto de la inclusión de Algamac 2000[®], Algamac Protein Plus[®], alga *Spirulina* en polvo, así como Dunalliella Gold[®], observando un mejor crecimiento alimentando a los juveniles con Algamac Protein Plus[®] o una mezcla 1:1 de ese suplemento con Algamac 2000[®]. Estos suplementos están preparados a base de microalgas para ser usados como alimento de larvas de crustáceos y peces, con la característica de ser ricos en DHA, fosfolípidos y carbohidratos.

El uso extendido de *S. thunbergii* en la alimentación de juveniles de *A. japonicus* ha provocado su sobreexplotación reduciendo su disponibilidad e incrementando su costo. Liu *et al.* (2010a) estudiaron el efecto de sustituir a *S. thunbergii* en su dieta empleado *Sargassum polycystum* así como talo o rizoma de *Laminaria japonica*, en una mezcla 80:20 de alga con lodo marino o suelo amarillo secos. Observaron que los organismos

alimentados con las dos especies de *Sargassum* presentaron una mayor eficiencia alimenticia y mejor crecimiento, aunque los resultados no fueron estadísticamente diferentes a los obtenidos con *L. japónica*. Si bien las especies de *Sargassum* presentaron un mayor nivel proteico, la tasa de ingestión de los animales alimentados con *Laminaria* fue mayor, compensando la diferencia en la ingesta de nutrientes.

Por otro lado, Martínez-Milián *et al.* (2016) en un estudio para identificar la mejor estrategia de alimentación para juveniles de *I. badionotus*, evaluaron mezclas 70:30 (arena de playa: alga) de las algas *Macrocystis pyrifera* o *Solieria filiformis*. Las algas se probaron en harina o en composta. No vieron diferencias en el uso de harinas o material predigerido en composta, y los mejores resultados se obtuvieron con *S. filiformis*, lo cual es explicado por el hecho de que esta alga posee mayor nivel proteico y un mejor perfil de aminoácidos y ácidos grasos en comparación a *Macrocystis*, por lo que se considera que tiene un mejor valor nutricional, además de ser un alga roja abundante en la zona de distribución de *I. badionotus*, donde forma parte de su alimentación natural.

El uso de subproductos agrícolas o agroindustriales se ha extendido en la alimentación de *A. japonicus*. Seo *et al.* (2011a) prepararon dietas a base de harinas de soya, *S. thunbergii*, *Undaria pinnatifida*, *L. japonica*, *B. oleracea*, así como subproductos de soya fermentada o de granos de destilería, polvo de paja de trigo y harina de trigo. Observaron el mejor desempeño con dietas a base de subproductos de fermentación de soya o con granos de destilería, lo cual atribuyen a los hábitos alimenticios del pepino de mar, donde consume detritos de materia orgánica mezclados con sedimento, que incluye microorganismos degradadores y sus enzimas, lo cual le ayuda a su proceso digestivo, además de que el sabor de la materia orgánica predigerida podría estimular su ingesta por tener un sabor parecido al de su alimento natural.

Tabla 2. Uso de alimentos completos e ingredientes para la formulación de dietas para pepino de mar

Especie	Etapa	Alimento	Comentarios	Referencias
<i>H. scabra</i>	Juvenil (1.7 mm)	Algamac 2000 [®] , Algamac Protein Plus [®] , alga <i>Spirulina</i> , <i>Dunaliella</i> Gold [®]	Mejor crecimiento con Algamac Protein Plus o una mezcla 1:1 con Algamac 2000	Giraspy & Ivy (2008)
A. <i>japonicus</i>	Juvenil (6.7 g)	<i>S. thunbergii</i> , <i>S.</i> <i>polycystum</i> , <i>L. japonica</i> (talo y rizoma), lodo marino, suelo amarillo	Mezcla 80:20 de harina de alga y lodo o suelo; mejor resultado con <i>Sargassum</i> .	Liu <i>et al.</i> (2010a)
<i>I.</i> <i>badionotus</i>	Juvenil (crecimiento: 0.24 g; alimentación 3.3g)	<i>Macrocystis pyrifera</i> y <i>Solieria filiformis</i> en harina o composta, arena de playa	Mejores resultados con <i>Solieria</i> en cualquier presentación con respecto a <i>Macrocystis</i>	Martínez-Milian <i>et al.</i> (2016)
A. <i>japonicus</i>	Juvenil (1.5 - 1.6 g)	Harinas de soya, <i>S.</i> <i>thunbergii</i> , <i>Undaria</i> <i>pinnatifida</i> , <i>L. japonica</i> , <i>B. oleracea</i> , de soya fermentada, de granos de destilería, polvo de paja de trigo, harina de trigo	Mejor crecimiento con dietas a base de harina de soya fermentada o con subproductos de destilería	Seo <i>et al.</i> (2011a)
A. <i>japonicus</i>	Juvenil (0.8 g)	Formulaciones a base de harinas de pescado, soya, ajonjolí, residuos de fabricación de tofu, harina de trigo, <i>S.</i> <i>thunbergii</i>	Mejor crecimiento con la mezcla de h. de pescado y <i>Sargassum</i> , con harina de soya y con la mezcla de todas las harinas	Seo <i>et al.</i> (2011b)
A. <i>japonicus</i>	Juvenil (0.9 g)	Harina de maíz, harina de soya extruida, <i>S.</i> <i>thunbergii</i> , harina de pescado, proteína de	El mejor crecimiento sustituyendo 20% de <i>S.</i> <i>thunbergii</i> con una mezcla 80:20 de harina de maíz y	Yu <i>et al.</i> (2015)

levadura, polvo de conchas, lodo marino, premezclas de vitaminas y minerales	de soya extruida, y hasta el 40% para una mejor rentabilidad del cultivo.
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En otro estudio, Seo *et al.* (2011b) evaluaron dietas formuladas a base de distintas fuentes proteicas (Tabla 2). No observaron diferencias en el crecimiento al alimentar con dietas preparadas con harina de pescado mezclada con *S. thunbergii*, con harina de soya como único ingrediente proteico, o con una mezcla de harinas de pescado, soya, ajonjolí, residuos de fabricación de tofu, harina de trigo y el alga *S. thunbergii*, por lo que recomiendan alimentar a este organismo con dietas a base de harina de soya.

Yu, Gao, Dong, Wen, Hou & Ning (2015) consideran que dada la sobreexplotación de *S. thunbergii*, el uso de subproductos agrícolas es la mejor alternativa para la preparación de alimentos formulados para *A. japonicus*. Ellos observaron que dietas donde se sustituye 20% del alga con una mezcla 80:20 de harina de maíz y de soya extruida, dan mejores resultados en crecimiento que la dieta a base de *Sargassum* como ingrediente principal, considerando que el nivel de sustitución puede llegar al 40% para una mejor eficiencia económica.

De acuerdo con la literatura disponible, se requiere formular dietas completas que permitan el cultivo intensivo de pepino en sistemas controlados, sin embargo, la formulación es complicada dados los hábitos alimenticios del organismo incluyendo su tendencia a seleccionar el alimento por tipo y tamaño de partícula. Hasta el momento no se dispone, al menos fuera de China, de dietas completas que satisfagan adecuadamente los requerimientos nutricionales de los organismos.

Hay un uso extendido de suplementos alimenticios desarrollados para la larvicultura de otros organismos acuáticos, y la tendencia para el uso de dietas completas es hacia la utilización de insumos de origen vegetal en sustitución a *S. thunbergii* que ha sido el alimento tradicional, aprovechando que el pepino de mar siendo un organismo detritívoro, consume en mayor medida detritos de origen vegetal. La mayoría de las investigaciones

apuntan hacia el uso extendido de harina de soya como ingrediente proteico, seguida de subproductos agroindustriales de bajo costo que permitan una mejor rentabilidad del cultivo.

La mayor parte de los estudios para la identificación de ingredientes y formulación de dietas se han hecho con juveniles para utilizarse en la etapa de criadero, habiendo una ausencia de información sobre el desarrollo de dietas completas para crecimiento y engorda en sistemas intensivos, así como para mantenimiento de reproductores, lo cual se considera una limitante para impulsar el cultivo de este organismo en sistemas controlados.

3. Prácticas de alimentación

3.1. Alimentación de larvas

En la acuicultura, los aspectos relacionados a la nutrición y alimentación de las especies en cultivo constituyen a los factores externos de mayor importancia, ya que el crecimiento y desarrollo de cada fase de vida, depende de un adecuado suministro y asimilación de nutrientes de cada etapa inmediata anterior. En el caso de los holoturoideos, el desarrollo embrionario es similar independientemente de la especie, hasta la formación de una gástrula planctónica, a partir de la cual hay dos rutas para el desarrollo de juveniles de vida bentónica; la primera es el desarrollo indirecto, en donde la gástrula continúa el desarrollo hacia la larva llamada auricularia, la cual sufre una metamorfosis hacia la larva doliolaria, que es la fase inmediata anterior al asentamiento y se presenta generalmente en las familias Holothuridae, Stichopodidae y Synaptidae (Hamel, Hidalgo & Mercier 2003; Sewell & McEuen 2006; Hu, Xu, Wen, Zhang, Fan & Su 2010). La segunda ruta es el desarrollo directo, en la cual, la gástrula da lugar a la larva doliolaria sin presentar la larva auricularia, estrategia dominante ya que ocurre en 22 de las 25 familias de holotúridos, generalmente en especies con huevos mayores a 150 micras de diámetro (Sewell & McEuen 2006), sin embargo, debido a que las investigaciones se han enfocado a especies comerciales, en especial *A. japonicus*, que presentan desarrollo indirecto, y por lo mismo las técnicas de cultivo están más avanzadas, los aspectos nutricionales que a continuación se discuten se enfocarán en estas especies.

La alimentación de los holotúridos durante el periodo larvario es inicialmente planctotrófica, constituida principalmente de fitoplancton, bacterias, zooplancton o detritus disponibles en la columna de agua (Levin & Bridges 1995; Qiu, Zhang, Hamel & Mercier 2015). De acuerdo con Sewell & McEuen (2006), la larva auricularia presenta tres estadios de desarrollo diferenciados como: auricularia temprana, auricularia intermedia y auricularia tardía, en relación con el desarrollo de estructuras del cuerpo y en longitud (<1mm), aspecto importante ya que en cada etapa ingiere alimento en distinta cantidad y tamaño de partícula.

La larva auricularia tiene una capacidad de nado limitada utilizando bandas ciliares presentes en el cuerpo, por lo que se alimenta pasivamente de partículas suspendidas en la columna de agua. Su sistema digestivo consiste en una boca rodeada de cilios, cuyo movimiento permite dirigir el alimento hacia la cavidad bucal, seguida de un esófago que con contracciones peristálticas permiten la ingestión del alimento hacia el bulbo estomacal y un intestino de longitud variable, con un recto que finaliza en el ano (Sewell & McEuen 2006).

La larva doliolaria continúa siendo planctónica, pero el tamaño es menor en comparación con la anterior fase auricularia (Hamel, Conand, Pawson & Mercier 2001; Laxminarayana 2005). Este estadio larvario constituye una etapa de transición, en el cual la larva sufre una metamorfosis para pasar de la vida planctónica a la bentónica, durante la cual no se alimenta, por lo que su desarrollo depende de la energía acumulada en estructuras de reserva denominadas esferas hialinas, que se desarrollan durante la etapa de auricularia tardía. Cabe mencionar que es en esta etapa donde suele ocurrir la mayor mortalidad en los criaderos (Asha & Muthiah 2002), debido a que ocurren cambios en el tracto digestivo que incluyen su reabsorción y modificaciones en la posición de las aperturas bucal y anal que se desplazan hacia los extremos para dar lugar a un estadio larval bentónico o pentáctula (Hamel, Hidalgo & Mercier 2003; Sewell & McEuen 2006; Al Rashdi, Eeckhaut & Claereboudt 2012).

La pentáctula se caracteriza entonces por la pérdida de la capacidad de nado en la columna de agua (Laxminarayana 2005; Guisado, Carrasco, Díaz-Guisado, Maltrain & Rojas 2012;

Hamel *et al.* 2001; Sewell y McEuen 2006), transformándose en un organismo asociado al fondo, donde su alimentación depende del consumo de las microalgas presentes en el sedimento, utilizando los primeros cinco tentáculos orales para colectarlas e ingerirlas. A partir de este momento presenta cambios morfológicos para dar lugar al juvenil (Sewell & McEuen 2006), el cual presenta ya una alimentación más parecida al adulto, consistente en diatomeas bentónicas y materia orgánica en degradación.

Durante los primeros días de desarrollo posteriores a la fertilización y hasta la formación de la gástrula planctónica, la energía es obtenida a través de los nutrientes contenidos en el huevo (Levin & Bridges 1995), razón por la cual la capacidad de la auricularia para desarrollarse y completar las fases críticas de metamorfosis y asentamiento como juvenil, dependerá de un adecuado suministro de alimento tanto en calidad como en cantidad a partir de la abertura de la boca e inicio de la alimentación exógena, ya que sólo logran pasar estas transformaciones cuando son fisiológicamente competentes, independientemente de la especie, duración de la etapa larvaria y condiciones de cultivo (Chia 1977; James, Gandhi, Palaniswamy & Rodrigo 1994; Morgan 2001; Asha & Muthia 2002; Asha 2004; Asha & Muthiah 2006; Chia 1977; Morgan 2009; Liu, Yang, Hongsheng & Liu 2010a).

Durante el desarrollo larvario de los holotúridos se reconocen dos periodos de elevada mortalidad relacionados con la alimentación. El primero de ellos ocurre al momento de la apertura de la boca e inicio de la alimentación exógena (auricularia temprana), y el segundo durante la metamorfosis a doliolaria y pentáctula, donde se dan cambios estructurales para la alimentación y adaptación a la vida bentónica (Battaglione 1999; Ivi & Giraspy 2006). Para llevar a cabo el cultivo larvario en cautiverio, se han desarrollado numerosos protocolos de alimentación provenientes de experiencias de laboratorios de producción y evaluaciones experimentales, los cuales en su mayoría usan microalgas vivas (Agudo, 2006; Al Rashdi 2012; Mercier & Hamel 2013). No obstante y pese a que los resultados de estos estudios han dado luz sobre las combinaciones de microalgas y/o alimentos que permiten un completo desarrollo de las especies estudiadas (Knauer 2011; Ren, Liu, Dong & Pearce 2016), hasta el momento, ninguna dieta ha logrado homogenizar el cambio de fase, por lo que, independientemente de la especie y dieta utilizada, al final del periodo larvario hay una notable heterogeneidad en el grado de desarrollo de las fases larvarias, que

dificultan el suministro adecuado de alimento para cada etapa (Asha & Muthiah 2002; Hamel *et al.* 2003; Mercier, Ycaza & Hamel 2004; Agudo 2006; Morgan 2008; Morgan 2009; Al Rashdi *et al.* 2012).

Uso de microalgas vivas

Independientemente de la especie, la alimentación exógena inicia al segundo día posterior a la fertilización, con la apertura de la boca de la larva (James, Rajapandian, Gopinathan & Baskar 1994; Hamel *et al.* 2003; Ivy & Giraspy 2006; Qiu *et al.* 2015), momento en el cual se suele suministrar microalgas vivas, ya sean solas o en mezclas de las especies: *Isochrysis galbana*, *Chaetoceros calcitrans*, *C. muelleri*, *C. gracilis*, *Tetraselmis* sp., *Rhodomonas salina* y *Pavlova lutheri*, para las primeras fases (Tabla 3), así como *Nitzschia* sp., *Navicula* sp., *Amphora* sp., *Achnanthes* sp. o *Platymonas* sp., para inducir el asentamiento y alimentación de la pentáctula y juveniles tempranos (Tabla 4).

Tabla 3. Estrategias de alimentación de la larva auricularia

Especie	Microalga o alimento	Referencias
<i>Actinopyga echinites</i>	<i>I. galbana</i>	Chen <i>et al.</i> (1991)
<i>A. (Stichopus) japonicus</i>	<i>P. tricornotum</i> , <i>D. salina</i> , <i>C. simplex</i> , Levadura	Chen (2003)
	<i>C. muelleri</i> , <i>N. closterium minutissima</i>	Sun & Li (2014)
	<i>C. gracilis</i>	Ito & Kiramura (1997, 1998)
	<i>C. muelleri</i> , <i>N. closterium minutissima</i>	Li <i>et al.</i> (2010)
	<i>C. muelleri</i> , <i>P. tricornotum</i> , <i>D. salina</i>	Matsuura <i>et al.</i> (2009); Liu <i>et al.</i> (2010b)
	<i>I. galbana</i> , <i>P. lutheri</i> , <i>C. gracilis</i>	Yanagisawa (1998)
<i>Athyonidium chilensis</i>	<i>T. suecica</i>	Guisado <i>et al.</i> (2012)
<i>Australostichopus</i> (<i>Stichopus</i>) <i>mollis</i>	<i>C. muelleri</i>	Morgan (2008, 2009)
<i>Bohadschia marmorata</i>	<i>C. calcitrans</i> , Pasta	Laxminarayana (2005)

Espece	Microalga o alimento	Referencias
<i>Holothuria (Theelothuria) spinifera</i>	<i>I. galbana, C. calcitrans, N. salina</i>	Asha & Muthiah (2002)
	<i>I. galbana, C. calcitrans, P. lutheri, N. salina</i>	Asha & Muthiah (2006)
	<i>I. galbana</i>	Asha (2004)
<i>Holothuria atra</i>	<i>C. calcitrans</i> , Pasta	Laxminarayana (2005)
	<i>Tetraselmis</i> sp., Levadura, Microcápsula Frippak	Ramofafia <i>et al.</i> (1995)
<i>Holothuria leucospilota</i>	<i>I. galbana, C. muelleri, C. calcitrans, Tetraselmis</i> sp.	Dabbagh <i>et al.</i> (2011)
<i>Holothuria mexicana</i>	<i>Dunalliella</i> sp.	Lacalli & West (2000)
<i>Holothuria scabra, H. scabra</i> var. <i>versicolor</i>	Pastas de <i>I. galbana, T. weissflogii</i> y <i>Pavlova</i> sp.	Duy <i>et al.</i> (2016)
	<i>C. muelleri</i> ; pastas de <i>I. galbana, Tetraselmis</i> sp., <i>T. weissflogii, T. pseudonana, Pavlova</i> sp.	Duy <i>et al.</i> (2017)
	<i>I. galbana, C. calcitrans, N. acicularis</i>	Mazlan & Hashim (2015)
	<i>C. muelleri, C. simplex</i>	Mercier <i>et al.</i> (2000)
	Pasta de <i>I. galbana; N. oculata, Platymonas</i> sp.	Pitt (2004)
	<i>I. galbana, C. muelleri, C. calcitrans, R. salina, Tetraselmis</i> sp.	Agudo (2006)
	<i>I. galbana, C. muelleri, C. calcitrans, P. tricornotum, Nannochloropsis</i> sp.	Al Rashdi <i>et al.</i> (2012)
	<i>C. calcitrans, C. gracilis, T. chuii</i>	Battaglione & Seymour (1998)
	<i>I. galbana, C. muelleri, C. calcitrans, R. salina, T. chuii</i>	Battaglione (1999)
	<i>I. galbana, C. muelleri, C. calcitrans, P. lutheri, R. salina, T. chuii</i>	Ivi & Giraspy (2006)

Especie	Microalga o alimento	Referencias
	<i>I. galbana</i> , <i>T. gracilis</i> , <i>C. salina</i>	James <i>et al.</i> (1994b)
	<i>I. galbana</i> , <i>C. muelleri</i> , <i>C. calcitrans</i> , <i>P. salina</i>	Knauer (2011)
	<i>I. galbana</i>	Morgan (2001)
	<i>I. galbana</i> , <i>C. muelleri</i> , <i>C. calcitrans</i> , <i>C. gracilis</i> , <i>R. salina</i>	Pitt (2001)
	<i>I. galbana</i> , <i>P. lutheri</i> , <i>C. gracilis</i>	Yanagisawa (1998)
<i>I. badionotus</i>	<i>I. galbana</i> y <i>T. weissflogii</i> en pasta; <i>C. muelleri</i> , <i>Tetraselmis</i> sp.	Zacaría-Soto <i>et al.</i> (2013)
<i>P. californicus</i> ,	<i>Isochrysis</i> sp., <i>C. muelleri</i> , <i>C. calcitrans</i> , <i>P. lutheri</i> , <i>P. tricornotum</i> , <i>D. tertiolecta</i> , <i>T. suecica</i> , <i>T. pseudonana</i>	Ren <i>et al.</i> (2016)
	<i>Dunaliella</i> sp.	Lacalli & West (2000)
	<i>I. galbana</i> , <i>P. lutheri</i> , <i>D. tertiolecta</i>	Smiley (1986)
<i>Stichopus</i> sp.	<i>Dunaliella</i> sp., <i>Rhodomonas</i> sp., Levadura <i>Rhodotorula</i>	Hu <i>et al.</i> (2010)

Tabla 4. Alimento utilizado para la inducción a la metamorfosis y asentamiento, así como primera alimentación de juveniles tempranos de holotúridos.

Especie	Tipo de alimento	Referencias
<i>A. japonicus</i>	<i>Laminaria japonica</i> , <i>Sargassum thunbergii</i> , <i>Sargassum</i> sp.	Li <i>et al.</i> (2010)
<i>A. (s.) japonicus</i>	<i>Nitzschia closterium minutissima</i> , <i>Nitzschia</i> sp., <i>Navicula</i> sp., <i>Amphora</i> sp., <i>Achnanthes</i> sp.	Ito & Kiramura (1997, 1998)
<i>Athyonidium chilensis</i>	Algamac [®]	Guisado <i>et al.</i> (2012)
<i>Australostichopus mollis</i> , <i>Stichopus mollis</i>	<i>C. muelleri</i>	Morgan (2008)
<i>Bohadschia marmorata</i>	<i>C. calcitrans</i> , Pasta	Laxminarayana (2005)
<i>H. scabra</i>	Algamac, <i>Spirulina</i> , <i>Nitzschia</i> sp., <i>Navicula</i> sp., <i>Platymonas</i> sp., <i>Sargassum</i> sp., hojas de algas	Agudo (2006)
<i>H. scabra</i>	<i>Spirulina</i> , <i>Nitzschia</i> sp. <i>Navicula</i> sp., <i>Sargassum</i> sp.	Al Rashdi <i>et al.</i> (2012)
<i>Holothuria scabra</i>	Algamac [®]	Pitt (2001)
<i>Holothuria scabra</i> var. <i>Versicolor</i>	Algamac Protein Plus [®] , Algamac 2000 [®] , <i>Spirulina</i> , <i>Nitzschia</i> sp., <i>Navicula</i> sp., algas	Ivi & Giraspy (2006)
<i>Holothuria scabra</i> , <i>H. scabra</i> var. <i>Versicolor</i> , <i>H. (metriatyta) scabra</i>	<i>Nitzschia</i> sp., <i>Navicula</i> sp., <i>Spirulina</i> , Algamac [®] , Algamac 2000 [®] , <i>Sargassum</i> sp.	James <i>et al.</i> (1994a)
<i>Holothuria (Theelothuria)</i> <i>spinifera</i>	Algamac [®] , <i>Spirulina</i> , Diatomeas, <i>Sargassum</i> sp.	Asha & Muthiah (2002)
<i>Holothuria atra</i>	<i>C. calcitrans</i> , <i>Tetraselmis</i> sp., Microencapsulado, Levadura	Laxminarayana (2005)
<i>Holothuria atra</i>	Diatomeas	Ramofafia <i>et al.</i> (1995)
<i>Isostichopus badionotus</i>	<i>Thalassiosira weissflogii</i> (Pasta), Algamac [®]	Zacarías-Soto <i>et al.</i> (2013)
<i>Holothuria scabra</i>	<i>Thalassia hemprichii</i>	Mercier <i>et al.</i> (2000)

Alimentación durante la fase planctónica

Las microalgas constituyen el alimento ideal, ya que es parte de la dieta natural de las larvas, son fáciles de producir en laboratorio, tienen un tamaño adecuado y las células son de fácil digestión (James *et al.* 1994a; Pitt 2001; James 2004), teniendo como desventaja la necesidad de disponer en el criadero de espacio suficiente para su cultivo masivo, la posibilidad de contaminación y pérdida del cultivo, así como la necesidad de mantener los cultivos en fase exponencial para asegurar una adecuada calidad de las microalgas.

Para seleccionar las microalgas que provean una buena alimentación y nutrición a las larvas de holotúridos, se han realizado investigaciones para determinar la mejor microalga o combinación de microalgas que permitan un adecuado desarrollo, metamorfosis y asentamiento. En algunas especies como *H. scabra* (Knauer, 2011), *A. mollis* (Morgan, 2008), *P. californicus* (Ren *et al.* 2016), *H. spinifera* (Asha y Muthiah, 2006) y *A. (S.) japonicus*, las larvas alimentadas con dietas monoespecíficas a base de las diatomeas *C. muelleri*, *C. calcitrans* o *C. gracilis*, tuvieron un tamaño mayor, así como tasas de supervivencia y metamorfosis más altos que las alimentadas con otras microalgas, mientras que se menciona que las larvas alimentadas con los flagelados *I. galbana* o *P. salina*, no produjeron esferas hialinas, su reserva energética para la metamorfosis, por lo que no completaron el desarrollo hacia doliolarias (Smiley 1986; Battaglione 1999; Morgan 2008; Knauer 2011), recomendando que el uso de *I. galbana* debiera ser usada sólo como complemento, y nunca como único alimento (Xilin 2004).

Sin embargo, el uso de otro flagelado como *Rhodomonas salina* para alimentar larvas de *H. scabra* y *H. fuscogilva*, ha resultado en un buen crecimiento y supervivencia (Battaglione 1999), mientras que Chen, Hsu & Deng (1991), James *et al.* (1994a), Morgan (2001), Asha & Muthia (2006), así como Knauer (2011), mencionan que el uso del dinoflagelado *I. galbana* como dieta monoalgal para larvas de *H. scabra*, *H. spinifera* y *Actinopyga echinites*, produjo los mejores resultados durante las primeras etapas, recomendando el uso de *Chaetoceros* sp. a partir del séptimo día posterior a la fertilización, mencionando que de esta manera se obtienen mayores tasas de asentamiento y supervivencia (James *et al.* 1994a; Ito & Kitamura 1997; Asha 2004; Agudo 2006; Asha & Muthia 2006).

Un caso particular es el uso de *Tetraselmis* sp., ya que los resultados han mostrado que esta microalga no es adecuada para la alimentación de larvas de holotúridos, pese a su contenido nutricional, no permitiendo completar la metamorfosis en *H. atra* (Ramofafia, Gervis & Bell 1995) y *H. scabra* (Battaglione 1999), o dando uno de los resultados más bajos en crecimiento, supervivencia y metamorfosis en larvas de *P. californicus* (Ren *et al.* 2016). Por ejemplo, Asha & Muthiah (2006) mencionaron que el uso de *P. lutheri*, *T. chuii* y *N. salina* como alimento en larvas de *H. spinifera*, dio como resultado una inhibición del crecimiento y una alta proporción de larvas deformes a partir del quinto día posterior a la fertilización, lo que de acuerdo con Morgan (2008), es un indicativo de una mala alimentación, que a su vez compromete el desarrollo y metamorfosis. Los resultados de estos estudios evidencian que, para una adecuada alimentación larvaria, es importante considerar: la especie de holotúrido, los requerimientos nutricionales específicos para cada fase larvaria en cantidad y calidad, y el contenido nutricional y tamaño de la microalga a utilizar.

Considerando estos resultados en éstas y otras especies, varios autores concluyen que una mezcla de microalgas es más adecuada para el desarrollo de larvas, en la medida en que se amplían las opciones para que la larva pueda consumir un alimento con un perfil bioquímico más adecuado (Hart & Strathmann 1995; Ramofafia *et al.* 1995; Battaglione 1999; Ivy & Giraspy 2006; Knauer 2011), y que la presencia de esferas hialinas en los estadios avanzados de auricularia son un indicador de la competencia de las larvas para llevar a cabo la metamorfosis (Asha 2004; Morgan 2002; Ramofafia, Byrne & Battaglione 2003), ya que se ha demostrado una correlación positiva entre el número de esferas hialinas con el tamaño de las larvas, además de que al menos para *A. echinites*, sólo las larvas que tuvieron esferas hialinas sobrevivieron después del asentamiento (Chen *et al.* 1991). No obstante, Sewell & McEuen (2006) mencionan que algunas larvas llevan a cabo la metamorfosis y desarrollo hacia juvenil con éxito sin la presencia de dichas esferas, por lo que el rol de éstas podría tener un papel en el almacenamiento de elementos estructurales utilizados después de la metamorfosis.

En relación a la ración, en varios estudios han observado que una alta concentración de microalgas afecta los niveles de pH y amonio en la incubadora, lo que a su vez influye en la

habilidad de la larva para filtrar e ingerir el alimento, inhibiendo el crecimiento, el desarrollo y dando como resultado una baja supervivencia en *H. scabra* (Morgan 2001), *S. mollis* (Morgan 2002) y *H. spinifera* (Asha 2004), por lo que es necesario establecer la concentración de microalgas adecuada de manera específica (Tabla 4).

Estudios con larvas de *H. scabra* (James *et al.* 1994a; Battaglione 1999; Morgan 2001), *H. atra* (Ramofafia *et al.* 1995), *S. japonicus* (Ito 1995; Ito & Kitamura 1997) y *H. spinifera* (Asha 2004), demuestran que la falta de alimento o el exceso de éste, provocan un efecto negativo en el desarrollo, siendo la ración sugerida entre 10,000 y 20,000 cel/ml, con la cual se presenta una mayor supervivencia, cambio de fase y adecuado desarrollo del tracto digestivo. Esto concuerda con lo reportado por Kumara, Jayanatha, Pushpakumara, Bandara & Dissanayake (2003), quienes al usar una concentración de 40,000 cel/ml durante el cultivo larvario de *Pseudocolochirus violaceus* y *Colochirus quadrangularis*, tuvieron altas tasas de mortalidad. Sin embargo, se contraponen con lo reportado por Ivy & Giraspy, (2006), quienes tuvieron buenos resultados al mantener una concentración de alimento de 40,000 cel/ml en larvas de *H. scabra* var. *versicolor*, argumentando que esto se pudiera deber a la longitud mayor del estómago de las larvas de esta variedad, en comparación con *H. scabra*. No obstante, la ración de alimento varía dependiendo de la especie y estadio de desarrollo, siendo entre 10,000 y 40,000 cel/ml lo comúnmente utilizado de forma progresiva conforme avanza el desarrollo de las auricularias (Tabla 5).

Alimentación durante la fase bentónica

Debido al cierre de la boca durante la metamorfosis, las microalgas suministradas durante este periodo tienen como intención alimentar a las larvas menos desarrolladas, así como generar una película de alimento (biofilm) para inducir el asentamiento de las pentáctulas (Tabla 5) (Mercier, Battaglione & Hamel 2000; Li, Li & Kong 2010).

Diversos autores mencionan que el uso de diatomeas bentónicas y Algamac[®] inducen el asentamiento y metamorfosis de las larvas de los holotúridos (Asha & Muthiah 2002; Ivy & Giraspy 2006; Li *et al.* 2010), incluso en más de un 50% cuando se suministra una densidad

de diatomeas mayor a las 200,000 cel cm⁻² (Ito & Kitamura 1997), o en un 83% cuando se utilizaron un film de *Ulvela* u hormonas neurotransmisoras como dopamina, L-adrenalina y L-noradrenalina en larvas doliolaria de *A. japonicus*, que es la fase en la cual el sistema nervioso se desarrolla y responde a estos estímulos (Matsuura, Yazaki & Okino 2009). Otra tendencia de inducción al asentamiento fue la encontrada por Qiu *et al.* (2015), quienes observaron que una concentración de entre 150 y 500 mg L⁻¹ de sólidos suspendidos totales en la columna de agua, propició una mayor tasa de asentamiento que las concentraciones de 50-150 mg L⁻¹ y 300-500 mg L⁻¹, sin embargo, las diferencias no fueron estadísticamente distintas que el grupo control, sin la presencia de estos sedimentos.

Tabla 5. Raciones diarias de alimento para larvas de holotúridos durante la fase de auricularia temprana (A1), auricularia intermedia (A2), auricularia tardía (A3), doliolaria (D) y pentáctula (P).

Especie	Fase larvaria					Referencias
	A1	A2	A3	D	P	
<i>A. echinites</i>	10,000					Chen <i>et al.</i> (1991)
<i>A. japonicus</i>	10,000	25,000				Chen (2003)
		20,000				Li <i>et al.</i> (2010)
	20,000	30,000	40,000			Liu <i>et al.</i> (2010b)
	5000	15,000	30,000			Sun & Li (2014)
	5,000 - 50,000					Yanagisawa (1998)
<i>A. chilensis</i>			1,200			Guisado <i>et al.</i> (2012)
<i>S. mollis</i>		600 - 3,000				Morgan (2002)
<i>A. mollis, S. mollis</i>			300 - 3,000			Morgan (2008)
			600 - 6,000			Morgan (2008)
			2,000			Morgan (2009)

Fase larvaria						
Especie	A1	A2	A3	D	P	Referencias
<i>B. marmorata</i>	20,000 - 25,000					Laxminarayana (2005)
<i>H. atra</i>	20,000 - 25,000					Laxminarayana (2005)
<i>H. atra</i>	40,000 - 80,000					Ramofafia <i>et al.</i> (1995)
<i>H. scabra</i> , <i>H. (metriatyla) scabra</i> , <i>H. scabra</i> var. <i>versicolor</i>	20,000	20,000- 25,000	25,000- 40,000			Agudo (2006)
	20,000	20,000	40,000	---		Al Rashdi <i>et al.</i> (2012)
	3,926 - 10,000	7,853 - 20,000	11,779 - 30,000			Duy <i>et al.</i> (2016)
		40,000				Duy <i>et al.</i> (2017)
		15,000 - 35,000		Algamac Protein Plus [®] , algas degradadas, diatomeas <i>Nitzschia</i> sp. Y <i>Navicula</i> sp, y <i>Spirulina</i>		Ivi & Giraspy (2006)
	10,000	25,000	30,000			James <i>et al.</i> (1994a, b)
	20,000- 22,000	25,000 - 27,000	30,000 - 32,500	35,000 - 37,500	40,000	Knauer (2011)
	20,000	35,000				Mazlan & Hashi (2015)
		25,000				Mercier <i>et al.</i> 2000

Especie	Fase larvaria					Referencias
	A1	A2	A3	D	P	
	0; 10,000; 20,000; 40,000; 80,000					Morgan (2001)
	20,000	25,000	30,000	35,000	40,000	Pitt (2001)
	5,000 - 50,000					Yanagisawa (1998)
<i>H. leucospilota</i>	20,000	30,000	40,000			Dabbagh <i>et al.</i> (2011)
<i>H. mexicana</i>		5,000				Lacalli & West (2000)
<i>H. spinifera</i> , <i>H. (T.) spinifera</i>	20,000 - 40,000					Asha & Muthiah (2002)
	0; 10,000; 20,000; 40,000; 80,000					Asha (2004)
	20,000					Asha & Muthiah (2006)
<i>I. badionotus</i>	20,000	30,000	40,000			Zacarías-Soto <i>et al.</i> (2013)
<i>P. californicus</i>	15,000 - 20,000					Ren <i>et al.</i> (2015)
<i>S. californicus</i>	5,000					Lacalli & West (2000)

Uso de concentrados y microparticulados

El uso de concentrados de microalgas o microparticulados es una alternativa para evitar los costos asociados al mantenimiento, área utilizada y producción de microalgas vivas en un criadero. Estos alimentos ofrecen la ventaja de poder ser almacenados y la practicidad de su uso (Yanagisawa 1998; Mercier & Hamel 2013).

Diversos estudios comprueban la factibilidad de utilizar concentrados de microalgas como único alimento durante el ciclo larvario, permitiendo el crecimiento y altas tasas de supervivencia post metamorfosis (Zacarías-Soto *et al.* 2013; Duy *et al.* 2016, 2017). Sin embargo, tienen la desventaja de precipitarse al ser un alimento inerte, disminuyendo el tiempo en que están disponibles en la columna de agua para ser aprovechado por las larvas. Además, la película de alimento no consumido que se acumula en el fondo inicia un proceso de descomposición en el tanque (y/o sustratos en el fondo) afectando la calidad del agua, dando lugar también a la proliferación de bacterias y copépodos que pueden dañar a las larvas recién asentadas (Mercier & Hamel 2013; Mazlan & Hashim 2015).

Si bien la presencia de películas bacterianas parece afectar de manera negativa a las larvas planctotróficas, también se ha mencionado que uno de los inductores al asentamiento de larvas en metamorfosis es justamente la presencia de estos biofilms en conjunto con diatomeas, sin embargo, hacen falta estudios concluyentes al respecto, que describan las características benéficas y perjudiciales de las películas bacterianas a nivel bioquímico.

En un estudio realizado por Ramofafia *et al.* (1995), se observó que el uso de *Tetraselmis* sp. deshidratada, como alimento para larvas de *H. atra* como dieta monoespecífica o en combinación con levadura y microencapsulados, permitió a las larvas el desarrollo hasta doliolaria, sin embargo no hubo asentamiento y todas las larvas murieron, sin poder determinar si estos resultados se debieron a la degradación de la calidad del agua, o al hecho de que las larvas no fueron capaces de ingerir o digerir la micropartícula haciendo de esta combinación una dieta insuficiente.

En otras evaluaciones en las que se han utilizado larvas sin alimentar como control, se ha observado que, aunque las larvas no llegan a presentar la metamorfosis, sí presentan cierto grado de crecimiento (Morgan 2008), siendo incluso mayor que en larvas con un exceso de alimento (Morgan 2001; Asha 2004), por lo que se ha sugerido que las larvas de los holotúridos tienen la capacidad de asimilar materia orgánica disuelta en el medio de cultivo (osmotrofia), algo que ha sido comprobado en larvas de bivalvos (Manahan 1990), aunque no en larvas de holotúridos (Knauer 2011).

Factores relevantes de su nutrición para una buena supervivencia larvaria

Un factor poco mencionado relacionado con la supervivencia, es tiempo para el suministro de la primera alimentación. Sólo un estudio ha evaluado el efecto del retardo en la alimentación, para determinar el punto de no retorno (PNR) en larvas de *A. japonicus*. Sun & Li (2014) mencionan que la falta de alimento por más de 48 h después de la fecundación (df) compromete seriamente el éxito del cultivo, siendo el PNR determinado de 110.4 h df. Además, retardar la alimentación alargó los días del cultivo, afectando el asentamiento.

Debido a que en varios estudios se ha demostrado que es posible que una larva no se desarrolle pero se mantenga con vida por varios días, la supervivencia a través del tiempo es una variable no adecuada para evaluar la competencia de las larvas para llevar a cabo la metamorfosis (Morgan 2009), siendo que ésta puede ser menor al 10% (Ramofafia *et al.* 1995; Ito & Kitamura 1998; Mazlan & Hashim 2015) hasta el 90% antes de llevar a cabo dicho proceso (Yanagisawa 1998), y 38% las que se desarrollan hacia doliolaria (Mazlan y Hashim 2015). En cambio, se ha mencionado que el desarrollo de estructuras como las esferas hialinas, el desarrollo del estómago hacia una forma esférica y el crecimiento pudieran servir como indicadores del estado y competencia de las larvas (Morgan 2008; Sun & Li 2014).

En este sentido, Ren *et al.* (2016) realizaron un estudio para saber si existe una relación entre el contenido de ácidos grasos poliinsaturados de ocho microalgas y el crecimiento de las larvas, como posible factor determinante del éxito del cultivo, sin embargo, los resultados no fueron concluyentes, ya que se obtuvieron resultados similares al alimentar larvas de *P. californicus* con microalgas con perfil de ácidos eicosapentaenoico (EPA) y docohexaenoico (DHA) variados, notando un menor crecimiento cuando, independientemente del perfil, se trató de las microalgas *T. pseudonana* y *T. suecica*. Otro estudio con resultados similares fue el realizado por Ren *et al.* (2016), quienes mencionan que al alimentar larvas de *A. japonicus* con concentrados de microalgas con un alto contenido de ácido araquidónico (ARA) y docohexaenoico (DHA), registraron una mayor supervivencia de auricularias tardías. Sin embargo, al alimentar larvas con el concentrado comercial de *T. weissflogii*, que presenta un alto contenido de EPA y bajo contenido en

DHA, se obtuvo una mayor tasa de supervivencia post metamorfosis, por lo que se concluyeron que otros elementos como proteínas, carbohidratos, minerales y vitaminas, pudieran ser nutrientes de mayor importancia (Ren *et al.* 2016).

En el estudio realizado por Duy *et al.* (2016), comprobaron este punto al evaluar la relación entre el crecimiento de larvas de *H. scabra* y el contenido nutricional de concentrados de microalgas (Isochrysis 1800[®], Pavlova 1800[®] y Thalassiosira weissflogii TW1200[®], Reed Mariculture Inc., Campbell, CA, USA), encontraron una correlación negativa entre el crecimiento y asentamiento con el contenido de proteína (208.17 – 255.22 mg g⁻¹ peso seco) y el contenido de lípidos (44.4 – 95.4 mg g⁻¹ peso seco), y una correlación positiva entre el contenido de carbohidratos con el crecimiento, supervivencia y la competencia de las larvas para llevar a cabo la metamorfosis, concluyendo que son los carbohidratos los nutrientes de mayor importancia para la alimentación de larvas de esta especie de holotúrido.

3.2. Alimentación de juveniles y adultos

Después de las fases larvarias planctónicas, las holoturias se asientan en el sustrato y empiezan a alimentarse de la materia orgánica que les rodea. Se podría pensar que seleccionan zonas con alto contenido en materia orgánica, pero la elección del sitio tiene más relación con la propia diseminación larvaria y con la presencia de depredadores (Slater & Jeffs 2010), es por esto por lo que los juveniles se localizan principalmente en oquedades bajo piedras, corales, macroalgas y otras superficies cubiertas de microalgas bentónicas, de forma que pueden ocultarse y tener el alimento suficiente para su desarrollo (Cameron & Fankboner 1989).

Estos aspectos de su ecología son los que definen los pasos a seguir en el cultivo; de hecho, para el crecimiento de juveniles tempranos se utilizan placas que previamente han sido inoculadas para desarrollar películas de microalgas bentónicas a la vez que se añade Algamac[®] al agua de cultivo para reforzar su nutrición (Battaglione, Seymour & Ramofafia 1999; Ito & Kitamura 1997; James *et al.* 1994; Yang, Hamel & Mercier 2015). Cuando los

juveniles ya han desarrollado el patrón de pigmentación y tienen un tamaño aproximado a 2 cm ya pueden ingerir alimentos con partículas de mayor tamaño, y para esta fase ya es posible la inclusión de harinas de alguna especie de macroalga, ya sea como un ingrediente no mayoritario (Orozco-Almeda, Sumbing, Lebata-Ramos & Watanabe 2014; Xia, Yang, Li, Liu, Zhou & Zhang 2012; Xia *et al.* 2015a; Xia, Wang, Gao, Sun, Zhang, Ma & Liu 2015b; Martínez-Milián & Olvera-Novoa 2016), o como ingrediente base para formular una dieta (Tabla 6), como es el caso de Liu *et al.* (2010a), quienes probaron dos especies de *Sargassum*, así como talo y rizoma de *Laminaria japonica*. Aun cuando los resultados de eficiencia alimenticia y tasa de crecimiento específico fueron mejores para los tratamientos con *Sargassum*, no fueron estadísticamente diferentes a los obtenidos con *Laminaria*, cuyos resultados fueron mejores con el rizoma en comparación al talo, por lo que concluyeron que el rizoma de *L. japonica* podía considerarse como una alternativa al uso de *Sargassum*, ya que la gran demanda de esta macroalga hace que cada vez sea un recurso más costoso mientras que la disponibilidad de rizoma de *Laminaria* es abundante.

También para juveniles de *A. japonicus*, Xia, Yang, Li, Liu, Zhou & Zhang (2012) probaron diferentes especies de macroalgas y otros productos de origen vegetal, observando que las mejores TCE eran las conseguidas por los animales alimentados con *Ulva lactuca* y *L. japonica*. Por otro lado, diversos investigadores sugieren que al tratarse de animales detritívoros, estos deben presentar mejores tasas de crecimiento con dietas basadas en ingredientes predigeridos, como por ejemplo algas fermentadas o subproductos de destilería (Seo 2011a), las propias heces de los animales (Ramofafia, Foyle & Bell 1997), desechos de granjas de animales acuáticos (Yuan, Yang, Zhou, Mao, Xu, Zhang & Liu 2006; Slater, Jeffs & Carton 2009; Zamora & Jeffs 2011) o en sistemas de Acuicultura Integrada Multitrófica (AIM) (Paltzat, Pearce, Barnes & McKinley 2008; Qi, Wang, Mao, Liu & Fang 2013; Orozco-Almeda *et al.* 2014). Esto se debe a que estos alimentos presentan una mayor carga microbiana y la actividad enzimática de los microorganismos podría aumentar el valor nutricional de los ingredientes, ya que de esta forma los nutrientes estarían más disponibles para el animal (Jones 1975).

Tabla.6. Estrategias de alimentación utilizadas con juveniles de pepino de mar

Especie	Alimento	Referencias
<i>A. japonicus</i>	<i>S. thunbergii</i> , <i>S. polycystum</i> y fango marino	Liu <i>et al.</i> (2010a)
	Soya Fermentada	Seo <i>et al.</i> (2011a)
	Heces de bivalvo y polvo de algas 75:25	Yuan <i>et al.</i> (2006)
	<i>Laminaria japonica</i> hervida	Xia <i>et al.</i> (2012)
	<i>S. thunbergii</i> y fango marino 75:25	Xia <i>et al.</i> (2015b)
	Fango marino y <i>G. lemaneiformis</i>	Gao <i>et al.</i> (2011)
<i>H. scabra</i>	Algamac Plus® y Algamac 2000®	Gyrapsi & Ivi (2008)
	Arena y <i>S. thunbergii</i>	Agudo (2006)
	Camarón y mejillón	Alameda-Orozco <i>et al.</i> (2014)
<i>A. mollis</i>	Deshechos de granja de mejillón	Slater <i>et al.</i> (2007, 2009) Zamora & Jeffs (2011)

Por otro lado, se han realizado estudios que buscan dilucidar que macronutriente utilizan las holoturias como fuente de energía de forma preferencial, para de esta forma diseñar un alimento formulado que se adapte a sus características fisiológicas, por lo que se pretende definir los niveles óptimos de proteína y carbohidratos para dietas de pepino de mar (Xia *et al.* 2015a, 2015b; Ye *et al.* 2009), ya que los carbohidratos son un recurso abundante de bajo costo que al ser incorporado en la dieta mejora las características físicas de la misma proporcionándole estabilidad a la fórmula. Zacarías-Soto & Olvera-Novoa (2015) evaluaron el efecto de tres dietas comerciales para animales de granja o acuícolas, con diferentes niveles de proteína y lípidos, sobre el crecimiento y la composición de la pared corporal de adultos de *I. badionotus*, llegando a la conclusión que los animales alimentados con las dietas que contienen al menos 20% de proteína y bajos niveles de lípidos son capaces de mantener su composición bioquímica a pesar de que no haya un aumento en el

crecimiento. También en esta especie, trabajos propios sin publicar, demuestran que dietas con un nivel de carbohidratos 45%- 60% proporcionan un mejor crecimiento que dietas con mayor contenido proteico, y esto parece que es general en todas las especies en las que se ha estudiado. Por ejemplo, en el caso de *A. japonicus*, Xia *et al.* (2015a, 2015b), definen que los niveles óptimos de carbohidratos se sitúan en torno al 50%. Esto se relaciona directamente con la capacidad que tengan las holoturias para digerir y asimilar los nutrientes que se ofrecen en cultivo, ya que, del total de la energía suministrada por el alimento solo una parte es asimilable por el animal en función de la digestibilidad.

En esta dirección se han realizado estudios con *A. japonicus* para determinar la digestibilidad de varias macroalgas (Xia *et al.* 2012a, 2012b), ingredientes de diferentes orígenes (Liu *et al.* 2009; Seo *et al.* 2011a, b), en función del procesado de la dieta (Xia, Yang, Li, Liu, Xu & Rajkumar 2013a; Xia, Yang, Li, Liu, Zhang, Chen & Zou 2013b) y en dietas con diferentes proporciones entre macronutrientes (Xia *et al.* 2015b). Se han realizado también estudios para medir el aprovechamiento de las dietas suministradas a *A. mollis* (Zamora y Jeffs 2011, 2012) y también que macronutrientes son los más digeribles por el animal (Slater, Lassudrie & Jeffs 2011), indicando que son los carbohidratos, y no las proteínas, los que ofrecen más energía digerible y asimilable para las holoturias.

Por otro lado, se sabe que la carga microbiana que de forma natural poseen los animales acuáticos en su sistema digestivo, ayuda tanto a la digestión y a la asimilación de nutrientes, así como el efecto beneficioso de los microorganismos en el sistema inmune y la aplicación de esta herramienta para la acuicultura (Riquelme & Avendaño-Herrera 2003; Jobling 2016), es por esto que en los últimos años se ha estudiado la importancia de incluir probióticos en las dietas del pepino de mar (Yasoda *et al.* 2006). Así, se han realizado estudios en los que se ha evaluado el efecto de la adición de probióticos sobre el crecimiento, la respuesta inmune y la resistencia frente a enfermedades en *A. japonicus* (Sun, Wen, Li, Meng, Mi, Li & Li 2012; Zhao, Zhang, Xu, Mai, Zhang & Liufu 2012; Yan, Tian, Dong, Fang & Yang 2014), indicando que el uso de probióticos mejora significativamente el desempeño y condición del organismo, lo que conlleva una optimización del proceso productivo, así como la reducción del uso de antibióticos, con la repercusión medioambiental que conlleva.

Finalmente, otro aspecto para tener en cuenta en la nutrición de holoturias es la evidencia de otro mecanismo de absorción de nutrientes. Diversos estudios evidencian la presencia de enzimas digestivos en el árbol respiratorio, aunque no se ha esclarecido si esta actividad es propiamente de enzimas producidas en las células del árbol respiratorio o pertenecen a la microbiota propia de este órgano, aunque teniendo en cuenta trabajos en los que se demuestra la incorporación de nutrientes a través del árbol respiratorio (Aheran 1968; Fontaine & Chia 1968; Jaeckle & Strathmann 2012; Brothers 2015), cabría pensar que cualquiera que sea la procedencia de esa batería enzimática, ésta contribuye a la nutrición de las holoturias y sería un aspecto relevante al momento de la alimentación de juveniles en criaderos.

4. Conclusiones

Si bien la tecnología para el cultivo de pepino de mar se inició en China en los años 50s del siglo pasado, y a la fecha la producción doméstica de ese país proviene exclusivamente de actividades acuícolas, a nivel mundial las poblaciones silvestres están sobreexplotadas y varias incluso agotadas, para satisfacer la elevada demanda de este organismo en el SE asiático, sin que se hayan desarrollado o establecido tecnologías apropiadas para el cultivo comercial fuera de los países asiáticos.

Entre las principales limitantes para desarrollar el cultivo comercial del pepino de mar se encuentra el desconocimiento de los requerimientos nutricionales y en consecuencia, la carencia de alimentos formulados para la mayoría de las especies con potencial acuacultural alrededor del mundo, particularmente en ambientes tropicales, aspecto que es influenciado por los hábitos alimenticios detritívoros del animal, que dificultan la preparación de dietas prácticas experimentales para determinar tanto sus requerimientos como para identificar ingredientes apropiados que permitan la formulación de dietas que sean aceptadas y utilizadas eficientemente por el organismo.

La mayor parte de la información disponible sobre nutrición de pepino de mar se centra en la principal especie comercial, *A. japonicus*, y en menor grado en especies emergentes en Oceanía como *H. scabra* y *A. mollis*, sin embargo, esta información es incompleta y de difícil aplicabilidad en otras especies, por lo que en la mayoría de los casos solamente es de

utilidad como un indicador, para generar la correspondiente a especies locales. Un aspecto relevante a considerar es que durante el ciclo del cultivo se requieren alimentos para al menos tres fases de su desarrollo ontogénico, con dos tipos de dietas para la etapa larvaria, una para la fase planctónica y una más para la larva recién asentada en el fondo, siendo esta etapa crítica por la elevada mortalidad que se presenta en criadero ante la falta de alimentos apropiados. Una vez como organismo bentónico, se requieren alimentos para crecimiento, engorda y mantenimiento de reproductores, cada uno de los cuales, con características diferentes en composición, tamaño de partícula y niveles de nutrientes.

De acuerdo con la revisión realizada, el pepino de mar es un organismo con requerimientos nutricionales muy diferentes a los de la mayoría de los organismos acuáticos cultivados, con necesidades proteicas y lipídicas de alrededor del 20 y 2-3%, respectivamente, los cuales se consideran un reflejo del bajo nivel trófico en el que se desenvuelve, por lo que el reto de diseñar dietas apropiadas es aún mayor, puesto que se necesita cambiar de paradigma y observar que, dado el bajo nivel trófico que las holoturias, sus necesidades deben estar más próximas a animales herbívoros, considerando que sus hábitos alimenticios detritívoros le permiten utilizar eficientemente materia orgánica en degradación, con una dominancia por materia vegetal, lo que permite el incorporar en su dieta insumos de bajo costo como por ejemplo subproductos agropecuarios o agroindustriales, por lo que cabría dirigir esfuerzos hacia estudios de la microbiota asociada al sedimento y la presente en sus órganos digestivos, para identificar ingredientes aceptables y digeribles para el diseño de dietas, incluyendo suplementos que optimicen su eficiencia.

En resumen, el impulso del cultivo intensivo de pepino de mar requiere de mayores esfuerzos para resolver las lagunas del conocimiento que permitan disponer de alimentos adecuados y eficientes para las diferentes etapas de la operación, incluyendo la identificación de ingredientes con calidad nutricional y digestibilidad apropiados, uso de aditivos y atractantes que estimulen la ingesta, etc.

Es importante también ahondar en el uso de dietas naturales y artificiales para el desarrollo de protocolos de alimentación para larvas, que maximicen la supervivencia y producción de juveniles en criaderos, seguido de la generación de alimentos microparticulados para la

primera alimentación de juveniles tempranos, aspecto que permitiría optimizar la producción de crías sin depender de alimento natural, elemento fundamental para desarrollar métodos de cultivo intensivos en sistemas controlados en tanques o sistemas cerrados en tierra.

A pesar de hace más de 50 años que el pepino de mar empezó a ser cultivado en China, a escala global es un organismo emergente para la acuicultura, y dada la importancia que ha cobrado su captura y comercialización especialmente en países tropicales y templados, donde la sobreexplotación ha agotado las existencias silvestres, el desarrollo de paquetes tecnológicos de cultivo contribuirá a mejorar la economía y bienestar de las comunidades ribereñas.

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Culture of Marine Sciaenids in Low Salinity: an Opportunity for Expanded Aquaculture in Mexico

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Abstract

The Sciaenidae is a highly diversified family of fish with worldwide distribution across warm-temperate and tropical waters. Numerous sciaenids are cultured commercially in different regions of the world. Most sciaenids are marine, but many representatives are euryhaline, *i.e.*, capable of withstanding a wide range of environmental salinities. Surprisingly enough, the family encompasses 25 strict freshwater species within six genera. Evidence of the salinity tolerance of various species of sciaenids across the world is large enough to suggest that commercial culture of sciaenids in low salinity is possible. In Mexico, among finfish families subjected to fisheries in the Pacific Ocean, Sciaenidae contributes with the largest number of captured species (24 species), while 13 species of sciaenids are captured in the Gulf of Mexico and the Caribbean Sea. Besides the 37 species exploited in commercial fisheries, there are more representatives of this family in Mexico. For example, at least 30 species of sciaenids are found in the Gulf of California alone. Taking into account this richness of species, some have been evaluated as candidates for aquaculture, including totoaba (*Totoaba macdonaldi*), the Gulf corvina (*Cynoscion othonopterus*), and the shortfin corvina (*C. parvipinnis*). All three species have shown to be euryhaline. For *T. macdonaldi*, definite proof that it can be cultured to marketable size in low salinity is presented. The information herein presented provides opportunities for expanded aquaculture of sciaenids in Mexico, using a variety of water sources, such as brackish or diluted saltwater, rivers, dams, or ground waters, perhaps in locations in which commercial culture was not previously thought possible.

Keywords: *Aquaculture, Sciaenids, Euryhaline, Low salinity.*

I-Introduction

Many aquatic species are not capable of tolerating changes in water salinity. These species, referred to as stenohaline (Gr. *stenos*: narrow + *halinos*: saline), are restricted to live in specific environments, for example either salt or freshwater. Conversely, some are capable of withstanding an ample range of environmental salinities, and are thus named euryhaline (Gr. *eury*: wide, broad + *halinos*: saline). Euryhalinity is of great interest from various perspectives, including the physiological, biological, and evolutionary. Furthermore, euryhalinity may have important practical implications, for example, for the rearing of marine organisms in low-salinity environments, such as brackish or diluted saltwater, as well as river, dam, or ground waters, in which commercial culture was not previously thought possible. The Pacific white shrimp, *Litopenaeus vannamei*, and the black tiger shrimp, *Penaeus monodon*, are excellent examples that the commercial culture of marine, euryhaline organisms is possible in unorthodox, low-salinity environments. Because of their amazing capacity to grow and survive in low salinity waters, *L. vannamei* has been cultured in groundwaters in the states of Alabama, Texas, Arizona, North Carolina, and Florida in the United States, as well as in other countries, such as China, Ecuador, Brazil (Roy *et al.* 2010), and Mexico (Perez-Velazquez *et al.* 2009). In turn, significant productions of *P. monodon* have been achieved in Thailand using brine solution obtained from saltwater evaporation, which is added to freshwater ponds to adjust salinity to values typically ranging from 2 to 5 g L⁻¹ (Flaherty & Vandergeest, 1998; Flaherty *et al.* 2000).

Euryhalinity also has been reported to occur in finfish, with the best documented examples represented by sciaenids. Commonly known as weakfishes, drums, and croakers, sciaenids are sport and food fish found around the world in warm-temperate and tropical waters (Chao, 1986; Sasaki, 1989). Sciaenid species from the genus *Cynoscion* spp. are sometimes named corvine/corvina (Lat. *corvinus*, from *corvus*: raven), because their lower jaw protrudes slightly beyond the upper jaw, resembling a raven's beak. During reproduction, sciaenids usually seek out estuarine areas where they encounter waters of lower salinity to spawn (Acha *et al.* 1999). For instance, it is well documented that the Gulf corvina, *Cynoscion ohtonopterus*, forms seasonal aggregations in the Colorado River Delta,

upper Gulf of California, Mexico, where they encounter lower salinities and spawn (Rowell *et al.* 2005). In many instances, juveniles spend the first stages of their life cycle in these environments until they are ready for recruitment and migrate back to the sea. Studies of these estuarine-dependent reproductive patterns of sciaenids indicate that, depending on the species, they can tolerate environmental salinities ranging from 5 to 42 g L⁻¹ (Cárdenas, 2012).

Sciaenids are cultured in different regions in the world, with an overall production of ca. 124,000 metric tonnes recorded in 2008 (FAO, 2012). The most widely cultured sciaenid species are the yellow croaker, *Pseudosciaena crocea*, the red drum, *Sciaenops ocellatus*, and the meagre, *Argyrosomus regius* (Cárdenas, 2012). Among these, *S. ocellatus* provides an excellent indication that culture of sciaenids is possible in low salinity. Although it is extensively cultured in seawater in the coasts of both the Atlantic Ocean and Gulf of Mexico in the United States, it has been documented that it may grow well in low-salinity brackish waters, as long as chloride concentrations exceed 130 mg L⁻¹ (Miranda and Sonski, 1985; Matlock, 1990; Wilson, 1990). Because of their firm, mild-flavored white meat, sciaenids are highly appreciated as food fish in local and international markets.

The present manuscript describes recent advances in low-salinity culture of fish belonging to the Sciaenidae family in Mexico.

II-Biogeographical notes on the Sciaenidae

The Sciaenidae is a highly diversified family of fish with worldwide distribution across warm-temperate, tropical, and typically not cold waters. It is one of the eight largest families within the Order Perciformes (Nelson, 2006). The Sciaenidae groups together 66 genera and 291 species (Eschmeyer & Fong, 2013). Based upon the classification of the world's oceans into four major basins, Eastern Pacific, Western Atlantic, Eastern Atlantic, and Indo-West Pacific (Springer, 1982), the greatest diversity of genera and species of sciaenids is found in the Indo-West Pacific basin, with 93 species. It is followed by the Eastern Pacific and Western Atlantic basins, of equal species richness in relation to one another, each with 82 species. Finally, the Eastern Atlantic basin is the least diverse, with

19 species (Lo *et al.* 2015). Most sciaenids are marine, although many species are known to inhabit brackish waters of estuaries and coastal lagoons. Surprisingly enough, the family encompasses 25 strict freshwater species within six genera. They are particularly important as a testament to the euryhaline nature of fish belonging to this family. The vast majority of these freshwater sciaenids, 23 species in four genera, are endemic to the Amazon basin and other parts of South America, one species is located in North America (*Aplodinotus grunniens*), and one in Southeast Asia (*Boesemania microlepis*) (Table 1) (Sasaki, 1989; Lovejoy *et al.* 2006; Lo *et al.* 2015). The evolutionary incursion of the Sciaenidae, and other freshwater fish species of marine lineages, from the marine to the freshwater environment has fascinated researchers for a long time. Ironically, the presence of strict freshwater sciaenids in different continents is not associated with a common inland freshwater ancestry prior to the breakup of Gondwana in the cretaceous period. Instead, it is believed that sciaenids invaded the freshwater habitat much more recently in three independent events in the Miocene.

Table 1. Geographical distribution of strict freshwater genera of the Sciaenidae.

Genera	Distribution	Source
NORTH AMERICA		
<i>Aplodinotus</i> sp.	United States, Mexico.	Sasaki, 1989
SOUTHEAST ASIA		
<i>Boesemania</i> sp.	Thailand, Vietnam, Cambodia, and Sumatra.	Kottelat <i>et al.</i> 1993
SOUTH AMERICA		
<i>Plagioscion</i> sp.	From northern Venezuela to lowlands of Argentina.	Chao, 1978
<i>Pachypops</i> sp.	From northern Venezuela to lowlands of Argentina.	Chao, 1978
<i>Pachyurus</i> sp.	From northern Venezuela to lowlands of Argentina.	Chao, 1978
<i>Petilipinnis</i> sp.	Brazilian Amazon basin, Cuyuni and Essequibo river basins in Guyana	Casatti, 2002

The South American incursion of the lineages *Plagioscion*/*Pachyurinae* is believed to have occurred 21 million years ago (Ma). The Caribbean Sea is considered the most likely point of entry, with transgressions extending into the upper Amazon basin. In North America, the freshwater invasion by *Aplodinotus* is hypothesized to have occurred 19.5 Ma, and by

Boesemania in Southeast Asia close to 10.3 Ma (Lovejoy *et al.* 2006). The idea that sciaenids must have performed a gradual marine-to-freshwater adaptation (Cooke *et al.* 2012) has been objected in favor of a recent theory of ecological fitting, *i.e.*, a common sciaenid ancestor/lineage must have already been capable of tolerating freshwater, facilitating the colonization of this environment (Boeger *et al.* 2015).

III-The Sciaenidae of Mexico: important fisheries species and candidates for aquaculture

Fisheries species of sciaenids are captured seasonally in Mexico along the coasts of the Pacific Ocean and the Gulf of Mexico. Genera exploited include *Atractoscion*, *Menticirrhus*, *Sciaenops*, *Micropogonias*, *Parolonchurus*, *Cynoscion*, *Leiostomus*, *Bairdiella*, *Larimus*, and *Cheilotrema*, to mention a few. Among finfish families exploited for fisheries in the Pacific Ocean, Sciaenidae contributes with the largest number of captured species with a total of 24 species, while 13 species of sciaenids are captured in the Gulf of Mexico and the Caribbean Sea (Fuentes-Mata and Espinoza-Pérez, 2010). In general, sciaenids are subjected to artisanal fishing and official fisheries statistics are fragmentary. The main exploited species by volume in the Pacific Ocean is the Gulf corvina, *Cynoscion othonopterus*, endemic to the Gulf of California, with a peak production of 6,000 tons in 2002, which decreased to 3,727 tons in 2010 (SAGARPA, 2012). The bigeye croaker, *Micropogonias megalops*, also endemic to the Gulf of California, is the second most important species, with 2,000 tons landed in 2015. Other sciaenids, also appreciated as game and food fishes in this region, include the shortfin corvina, *Cynoscion parvipinnis*, *C. nobilis*, *C. reticulatus*, *C. xanthulus*, *Atractoscion nobilis*, *Bairdiella incista*, *Cheilotrema saturnum*, *Larimus acclivis*, *Menticirrhus nasus*, *M. panamensis*, *M. undulatus*, *Roncador stearnsii*, *Umbrina roncador*, and *U. xanti* (Gobierno del Estado de Baja California, 2000-2015). In the Gulf of Mexico, fish landings of *Cynoscion arenarius*, *C. nebulosus*, and *C. nothus* collectively amounted to ca. 3,000 metric tonnes in 2008 (SAGARPA, 2012). Other sciaenids associated with these fisheries, but captured in smaller quantities, are *Bairdiella chrysoura*, *B. ronchus*, *Menticirrhus americanus*, *M. littoralis*, *M. saxatilis*, *Micropogonias undulatus*, *Pogonias cromis*, and *Sciaenops ocellatus*

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(SAGARPA, 2012). In addition, the geographical distribution of the only North American strict freshwater sciaenid, *Aplodinotus grunniens*, extends from the United States, including the Rio Bravo in the Atlantic basin, to the Usumacinta-Grijalva River in southeastern Mexico (Espinosa-Perez *et al.* 1993). Highly appreciated as food fish, this species is of local economic importance in some dams, such as “Malpaso” in the state of Chiapas (Anzueto-Calvo *et al.* 2013).

The development of technology for the commercial culture of the sciaenids *P. crocea*, *S. ocellatus*, and *A. regius* in Asia, the Americas, and Europe, respectively, is *fait accompli* (Cárdenas, 2012). Taking into account the richness of species of sciaenids found in Mexico, mass culture of sciaenids may soon become a reality. Several candidate species have been identified. Without doubt, totoaba, *Totoaba macdonaldi*, is a prominent candidate species. The story of totoaba is a fascinating one. It is endemic to the Gulf of California and a true giant, the largest representative of the family Sciaenidae, reaching up to 2 m (6.5 feet) in length and 135 kg (298 lbs) in weight (Flanagan and Hendrickson, 1976). *T. macdonaldi* supported a prosperous fishery in the upper Gulf of California in the 1950s (Bobadilla *et al.* 2011). Due to overfishing, it was catalogued as an endangered species and fishing was completely banned since 1975 (Mexican Official Norm NOM-059-ECOL-1994). Reproduction of totoaba in captivity was first accomplished at Universidad Autonoma de Baja California (UABC) in Ensenada, Baja California, Mexico. Recently, a second successful breeding program was established for stock enhancement and aquaculture purposes at the Center for Reproduction of Marine Species of the State of Sonora (CREMES), Kino Bay, Sonora, Mexico. Since 2013, CREMES has released thousands of juvenile fish into the wild every year near Kino Bay, Gulf of California. Interestingly, captive rearing has shown that this species has very fast growth rates. With its highly appreciated meat quality, as well as the stratospheric prices its swim bladder fetches in specialty Chinese cuisine markets (Juarez *et al.* 2016), totoaba has been targeted for expanded aquaculture in Mexico. Currently, the private company Earth Ocean Farms conducts commercial growth trials in submersible cages near La Paz, Gulf of California. The results have been more than encouraging and have shown that totoaba can reach 2.5 kg of weight in one year and 6 kg in two years (Juárez *et al.* 2016).

Other sciaenid species, the Gulf corvina, *Cynoscion othonopterus* (Figure 1), and the

shortfin corvina, *Cynoscion parvipinnis* (Figure 2), have also been evaluated as candidates for aquaculture at the University of Sonora and other institutions in the northwestern region of Mexico. Using fingerlings and juveniles produced at CREMES, various nutritional aspects of these species have been evaluated. Although these studies have been conducted in short-term trials, 6 to 8 weeks, and at small scale in experimental tanks, it has been possible to show that growth rate of both species, although not as spectacular as that of totoaba, compares well with the average growth rates of other sciaenids of similar size (Perez-Velazquez *et al.* 2015; González-Félix *et al.* 2015; Minjarez-Osorio *et al.* 2016; González-Félix *et al.* 2016).



Figure 1. Gulf corvina, *Cynoscion othonopterus*.

The former examples of sciaenid species as candidates for aquaculture may be just the tip of the iceberg. In Mexico, there are more representatives of the family than the species described earlier as fisheries species. For example, at least 30 species of sciaenids are found in the Gulf of California alone (Van der Heiden, 1985). The vast majority of these species have not been evaluated for aquaculture. The possibilities of advancing the culture of sciaenids in the near future in Mexico is certainly promising.



Figure 2. Shortfin corvina, *Cynoscion parvipinnis*.

IV-Culture of sciaenids in low salinity: the evidence so far

Red drum, *S. ocellatus*, is an excellent example that euryhaline organisms can be reared successfully in low salinity. Forsberg *et al.* (1996) reported that red drum shows adequate growth rates in groundwaters at salinities between 5 and 15 g L⁻¹. It has also been reported that it may grow well in low-salinity brackish waters, as long as chloride concentrations exceed 130 mg L⁻¹ (Miranda and Sonski, 1985; Matlock, 1990; Wilson, 1990). Furthermore, in a 6-week feeding trial, weight gain of red drum was greater when reared at 5 g L⁻¹, as compared to full strength seawater (Craig *et al.* 1995). As additional proof of its ample tolerance to low salinity, 1.5 to 2 million red drum fingerlings are stocked annually for sport fishing into freshwater reservoirs in Texas, USA, such as Calaveras and Victor Braunig in the vicinity of San Antonio, as well as Tradinghouse Creek near Waco, and Fairfield, named after the town that bears the same name (Hodge, 2006). Because of the good response of red drum to low salinity, growth trials with this species are routinely conducted at salinity of ca. 7 g L⁻¹ at the Texas A&M Aquacultural Research and Teaching Facility of Texas A&M University, College Station, Texas, USA, which is preferred over natural seawater salinity (Rossi *et al.*, 2015; Minjarez-Osorio *et al.* 2016).

In Australia, approximately 2.5 million ha of land, formerly used for agriculture, have been affected by secondary salinization of groundwaters, which has sparked interest in using them for inland aquaculture (Doupé *et al.* 2003; Partridge *et al.* 2008). The mullet, a

Argyrosomus japonicus, has been targeted as one of the species of choice for this purpose in Australia, in view of its great potential for low-salinity inland culture (Doupé *et al.* 2003). *A. japonicus* is a sciaenid which is normally produced in sea cages (O'Sullivan *et al.* 2007), but adequate growth of this species in inland saline groundwaters was reported by Doroudi *et al.* (2006). In fact, *A. japonicus* has already been cultured commercially using saline inland water in that country (Partridge *et al.* 2008).

The yellow croaker, *Pseudosciaena crocea*, an economically important coastal species from Asia, and silver kob, *Argyrosomus inodorus*, also of economic importance in the southeastern Atlantic coast of Africa, add to the list of sciaenids known to tolerate low salinity well. Growth of these species is at least as adequate at low salinity as in natural seawater (Ferreira *et al.* 2008; Wang *et al.* 2016). For the Atlantic croaker, *Micropogonias undulatus*, growth observed in low salinity was greater than in full strength seawater (Peterson *et al.* 1999), similarly to reports for red drum (Craig *et al.* 1995).

In Mexico, the salinity tolerance of *T. macdonaldi*, *C. othonopterus*, and *C. parvipinnis*, has been investigated. All three species have been shown to be euryhaline, their isosmotic salinities were established at 12.3, 9.8, and 13.4 g L⁻¹, respectively. After 6 weeks of culture, growth rate of *C. othonopterus* was similar throughout the salinity range of 5 to 35 g L⁻¹ (Perez-Velazquez *et al.* 2014). For *T. macdonaldi* and *C. parvipinnis* reared during 8 weeks, somatic growth was greatest at 10 g L⁻¹ (González-Félix *et al.* 2017). Specific growth rate (SGR) observed at salinities of or above 10 g L⁻¹ for these species (0.9 to 2.3% d⁻¹) compared favorably to the range of SGRs, from 0.7 to 2.5% d⁻¹, reported for a number of other sciaenids, such as *S. ocellatus*, *A. japonicus*, *Sciaena umbra*, and *Umbrina cirrosa* (Sandifer *et al.* 1993; Jirsa *et al.* 1997; Segato *et al.* 2005; Chatzifotis *et al.* 2006). Because the previous studies were conducted at small scale and for a relatively short time, the authors warned that growth rates of *T. macdonaldi*, *C. othonopterus*, and *C. parvipinnis* in low salinity may be susceptible of improvement by using infrastructure of greater size and culturing fish preferably to market size. Therefore, a set of results on growth of *T. macdonaldi* reared to a larger size in low salinity (12 g L⁻¹) and in seawater (36 g L⁻¹) is presented below. The data represent the first report on growth of *T. macdonaldi* in low salinity to what may be considered ready-for-sale size in certain niche markets. The study was conducted at CREMES, Kino Bay, Mexico, using fish produced at this hatchery, which

were cultured in a recirculating aquaculture system for 115 days. Figure 3 shows growth of fish and mean water temperature. Body weight of fish cultured at low salinity slightly surpassed those held in seawater, in agreement with previous observations for this species (González-Félix *et al.* 2017). Many individual fish reached over 1 kg of weight at both salinities. On average, from an initial body weight of approximately 400 g, fish reached close to 900 g in 115 days of culture.

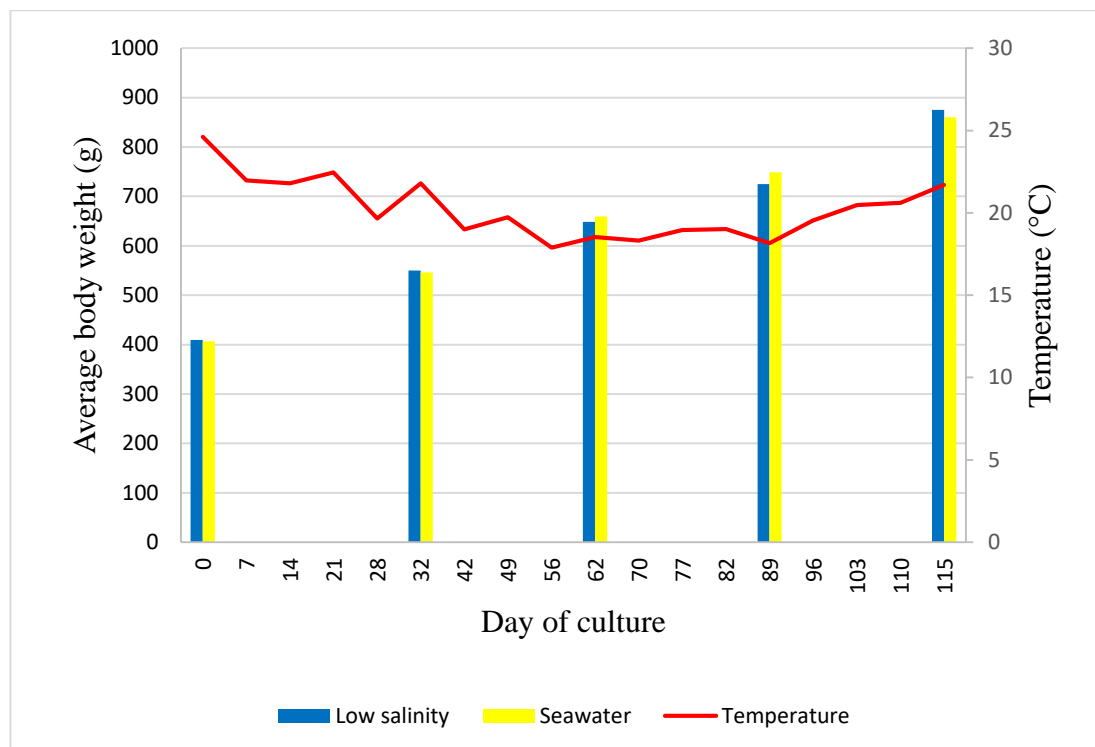


Figure 3. Growth of totoaba (*Totoaba macdonaldi*) reared for 115 days in a recirculating aquaculture system at low (12 g L^{-1}) and seawater salinity (36 g L^{-1}).

Growing at such rates, it is very likely that, if reared for a full year, fish could have matched the 2.5 kg of annual growth rate reported by Juárez *et al.* (2016) for totoaba held in seawater cages. Fish grown at low salinity were identical to the saltwater-reared counterparts and did not show any organoleptic differences after cooking. These results are definite proof that totoaba can be cultured to marketable size in low salinity.

V-Conclusions

The Sciaenidae is a highly diversified family of fish with a remarkable ability to withstand salinity changes. So far, the body of evidence gathered related to their culture in low salinity, in terms of the high number of species and their diverse geographical distribution, is overwhelming. In Mexico, with the advent of commercial seawater culture of totoaba in the Gulf of California, the herein presented proof that this species can be also grown to marketable size at low salinity provides opportunities for culture in a variety of water sources, such as brackish or diluted saltwater, rivers, dams, or ground waters, perhaps in locations in which commercial culture was not previously thought possible. Together with the available evidence of low-salinity tolerance of the Gulf corvina, *C. othonopterus*, and the shortfin corvina, *C. parvipinnis*, this information opens possibilities for advancing the knowledge concerning low-salinity culture of more representatives of the many species of sciaenids found in Mexico.

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Lípidos Alternativos en la Nutrición de Peces Marinos | Alternative Lipids in Nutrition of Marine Finfish

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Abstract

The current paper provides new knowledge regarding LC-PUFA requirements of marine carnivorous fish (White Seabass *Atractoscion nobilis*, California Yellowtail *Seriola dorsalis* and Florida Pompano *Trachinotus carolinus*) in the context of C₁₈ PUFA-rich and SFA- and MUFA-rich alternative lipids. Determine if all LC-PUFAs (ARA, EPA, DHA) are equally important in meeting fatty acids requirements and also determine the effects of dietary SFA, MUFA, and C₁₈ PUFA content in fish oil sparing and tissue deposition of LC-PUFAs. The overall findings highlighted that DHA and ARA appear to be the primary drivers of fatty acid essentiality, whereas EPA is likely required in minor amounts. It was also demonstrated that DHA/EPA ratio had little-to-no effect on fish performance. Additionally, LC-PUFA requirements seem to be more flexible than previously assumed being influenced by dietary fatty acid profile. LC-PUFAs in marine finfish are more bioavailable in the context of SFA-/MUFA-rich alternative lipids, thus, reducing the requirements for these nutrients and allowing the fish's physiological demand to be met with dietary levels below the minimum levels recommended. Finally, these findings suggest that although marine fish accept a variety of alternative lipids, those rich in SFAs and/or MUFAs seem advantageous in terms of limiting the effects of fish oil sparing on tissue fatty acid profiles.

Keywords: Lípidos, Peces marinos, Alimentos

Introduction

Fish oil sparing and replacement is considered one of the biggest challenges faced by the aquaculture industry and aquaculture nutrition because of the unique characteristics of this marine-origin lipid. The high digestible energy and abundance of long-chain polyunsaturated fatty acids (LC-PUFAs), particularly arachidonic (ARA, 20:4n-6), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic acids (DHA, 22:6n-3), which are essential nutrients for most carnivorous species, make fish oil an especially valuable ingredient for aqua feed manufacturing (Turchini *et al.* 2009). Consequently, aquaculture consumes most of the fish oil globally available (Tacon & Metian 2015). Growing demand in the context of a limited supply, as well as other factors, has caused the price of fish oil to increase. From 2010 to 2015 the price of fish oil increased from US\$ US\$1,000.00/million ton to US\$2,500.00/million ton (FAO 2015). Increasing cost has encouraged the aqua feed industry to look for alternative lipid sources to spare or replace this expensive, but otherwise fine ingredient (FAO 2015). Vegetable- and terrestrial animal-origin lipids contain little-to-none of the physiologically important LC-PUFAs. Instead, these lipids are rich in other fatty acids: vegetable oils are commonly rich in physiologically inactive C₁₈ polyunsaturated fatty acids (C₁₈ PUFAs; mainly 18:2n-6 and, in few oil types, 18:3n-3), whereas terrestrial animal lipids are rich in saturated fatty acids (SFAs, mainly 16:0) and monounsaturated fatty acids (MUFAs, mainly 18:1n-9) (Turchini *et al.* 2010). Assuming the essential fatty acid requirements of fish are met, fish performance is normally unaffected by dietary lipid source (Turchini *et al.* 2009). However, fish survival rates, growth, and production performance can be impaired as a result fish oil sparing when the diet does not provide adequate levels of essential fatty acids (Turchini *et al.* 2009). These negative effects are most likely to occur in carnivorous fish fed reduced or fish oil-free feeds, due to these species' dietary requirements for LC-PUFAs. In fact, complete fish oil replacement in carnivorous fish without growth impairment is usually only reported for feeds that have been amended with LC-PUFAs supplements or contain considerable residual marine-origin lipid from dietary fish meal. In addition to effects on fish performance, dietary inclusion of alternative lipids also alters the fatty acid profile of fish,

altering the composition and product quality of the edible tissues. It has been extensively reported that the fillet fatty acid profile and associated nutritional value typically reflect the composition of the dietary lipid (Turchini *et al.* 2009). Accordingly, further research on alternative sources of LC-PUFAs, and alternative lipid sources that maintain performance and tissue fatty acid composition are needed in aquaculture nutrition, particularly for carnivorous fish.

By understanding fatty acid essentiality of intended fish species, fish nutritionists will be able to further address the fish oil bottleneck in aquafeed manufacturing. However, determining fatty acids requirements is complex because they commonly vary among species according to trophic level and the environment in which they evolved, as well as within species according to the developmental stage and physiological condition (Sargent *et al.* 2002). Additionally, other external and internal factors, including basal dietary composition, feeding strategy, and rearing conditions are also known to influence fatty acid requirements (Sargent *et al.* 2002). There is growing evidence, for example, that C₁₈ PUFAs—long considered essential nutrients for all vertebrates—are physiologically inactive and serve only as precursors to the LC-PUFAs which possess true biochemical or physiological functionality (Sargent *et al.* 2002). Preliminary results suggest that not all LC-PUFAs (ARA, EPA, and DHA) may be equally required (Trushenski *et al.* 2012). DHA seems to be important, whereas EPA appears to be expendable in some species; ARA, apparently required in comparatively minor amounts, has simply not been investigated to the same extent as the n-3 LC-PUFAs (Trushenski *et al.* 2012). Additionally, it is becoming clearer that the fatty acid composition of dietary alternative lipid influences fatty acid bioavailability and the degree of tissue fatty acid distortion (Turchini *et al.* 2009). More specifically, alternative lipids rich in SFAs and/or MUFAs appear to influence LC-PUFA bioavailability and, in some instances, seem to enhance fillet LC-PUFA deposition. Clearly, the questions of essential fatty acid requirements of fish, especially marine carnivores, are multifold, complex, and interrelated. In this context, the current paper sought to provide new knowledge regarding LC-PUFA requirements of marine carnivorous fish in the context of C₁₈ PUFA-rich, SFA-, and MUFA-rich alternative lipids. Further, it sought to determine if ARA, EPA, and DHA are equally important drivers of fatty acid

essentiality in representative species. Finally, it sought to determine the effects of dietary SFA, MUFA, and C₁₈ PUFA content on tissue deposition of LC-PUFAs in marine carnivorous fish.

Results and Discussion

Marine carnivorous fish exhibit physiological demand for ARA, EPA, and DHA, but may not require each of them to be provided intact in the diet. Trushenski *et al.* (2012) demonstrated that DHA is essential, whereas EPA is largely expendable or required only in trace amounts in Cobia (a marine carnivore, trophic level = 4.0) feeds. These results contradicted the previous reported joint requirements for EPA and DHA, but similar results were repeatedly demonstrated, adding further credibility to these findings. I found similar results regarding the relevance of DHA in White Seabass (trophic level = 4.3) fed fish-oil free feeds. My study with California Yellowtail (trophic level = 4.2) provided further supporting information, emphasizing the greater relevance of DHA versus EPA, as well as demonstrating the essentiality of ARA. Furthermore, DHA appears to be more influential than EPA in the context of promoting growth of Florida Pompano (trophic level = 3.5). Based on these findings and previous results, I suggest that DHA and ARA appear to be the primary drivers of fatty acid essentiality in marine carnivorous fish with trophic level equal or higher than 3.5; EPA, if required, appears necessary only in trace amounts.

These findings draw a clear contrast with the reported requirements of lower trophic level species and, to a lesser extent, the requirements previously reported for marine carnivores. With respect to omnivorous and herbivorous species, it has been reported that they require 18:2n-6 and/or 18:3n-3 as essential fatty acids (NRC 2011). For example, Tilapia spp. require 18:2n-6 between 0.5-1.0% of the diet, Channel Catfish *Ictalurus punctatus* require 18:3n-3 between 1.0-2.0% of dry diet, and Carp spp. require both C₁₈ precursors between 0.5-1.0% of the diet (NRC 2011). For marine carnivores it has been highlighted the importance of n-3 LC-PUFAs (i.e. EPA + DHA) but not of n-6 LC-PUFAs (i.e. ARA) to fatty acid essentiality (NRC 2011). The reported EPA + DHA requirement of representative marine carnivorous species, including Cobia, *Seriola* spp., Turbot

Scophthalmus maximus, Grouper *Epinephelus* spp., Red Drum *Sciaenops ocellatus*, Red Sea Bream *Pagellus bogaraveo* and Gilthead Sea Bream *Sparus aurata* consist in a range between 0.5-3.9% of the the diet (NRC, 2011; Trushenski *et al.* 2012).

Dietary DHA/EPA ratio has been used to investigate n-3 LC-PUFAs in several marine species. This approach is seemingly based on the biosynthetic relationship between these two fatty acids and emphasizes the importance of the relative proportions of these two nutrients in the diet. However, DHA and EPA have distinct physiological roles and different degrees of importance in terms of fatty acid essentiality, undermining the relevance of examining these two nutrients in proportion to one another. Thus, perhaps understandably, it has been reported that DHA/EPA ratio had little-to-no effect on juvenile Cobia performance (Trushenski *et al.* 2012). Likewise, my results with juvenile California Yellowtail and Florida Pompano provide further evidence that DHA/EPA ratio is not a critical benchmark of proper feed formulation. In the California Yellowtail study, fish oil-free feeds supplemented with EPA and all LC-PUFAs (ARA, EPA, and DHA) at the 100% level had equivalent DHA/EPA ratios of 1.0. However, fish fed the former feed exhibited reduced growth performance; whereas those fed the latter feed exhibited improved performance. In the Florida Pompano trial, dietary treatments had DHA/EPA ratios ranging from 0.4 – 2.5, but none of these yielded significantly different fish performance. Dietary ratios of DHA to EPA seem substantially less important than the absolute dietary concentrations of both nutrients, at least in the context of fish with limited ability to bioconvert EPA to DHA. Further research regarding the subject of DHA/EPA ratios may be warranted, but the collective results of this dissertation suggest that a focus on absolute, independent requirements for these nutrients is likely to be a more successful strategy to formulate marine finfish feeds and use fish oil as judiciously as possible.

Requirements for LC-PUFAs seem to be more flexible than previously presumed. Specifically, it is increasingly apparent that such requirements being influenced by dietary fatty acid composition and the effect of overall dietary composition on the availability of LC-PUFAs. The present results demonstrated that fish oil sparing with C₁₈ PUFA-rich lipids is largely constrained by dietary availability of LC-PUFAs (mainly DHA and ARA).

In California Yellowtail, adequate dietary DHA and ARA supplementation in soybean oil-based feeds (high C₁₈ PUFA content) was necessary to completely replace fish oil without impairing production performance and reducing LC-PUFA content of fish tissues. Such supplements were also necessary in White Seabass feeds containing C₁₈ PUFA-rich soybean oil, but not those containing SFA-rich hydrogenated soybean oil. It was previously reported that alternative SFA-rich lipids are advantageous in respect of meeting LC-PUFA requirements of marine carnivorous fish fed reduced or fish oil-free feeds. These authors demonstrated that LC-PUFAs requirements of White Seabass were effectively reduced in the context of SFA-rich feeds. In my study with the same species, it was demonstrated that the level of DHA needed to satisfy the dietary requirement of White Seabass was lower in the context of SFA-rich feeds compared to C₁₈ PUFA-rich feeds. Similarly, I demonstrated that n-3 LC-PUFAs were more bioavailable and the presumptive LC-PUFAs requirements of Florida Pompano for EPA and/or DHA were reduced in the context of SFA- and MUFA-rich feeds. Recent research with California Yellowtail and Cobia fed SFA- and/or MUFA-rich feeds provide further evidence in support of the hypothesis that dietary LC-PUFAs are more bioavailable in the context of SFA- and/or MUFA-rich lipids, thus, reducing the requirements for these nutrients and allowing the fish's physiological demand to be met with dietary levels below the minimum levels typically recommended.

Similar to the effects of dietary fatty acid composition on LC-PUFA availability, it is becoming more evident that dietary fatty acid also influences tissue deposition of LC-PUFAs. In most cases, C₁₈ PUFA-rich feeds appear to reduce the availability and deposition of LC-PUFAs in tissues. Conversely, SFA-, and MUFA-rich feeds exhibit a less overt effect on tissue fatty acid composition and attenuate the loss of beneficial LC-PUFAs typically associated with fish oil sparing. I observed this so-called LC-PUFA sparing effect, in my research with White Seabass and Florida Pompano. Although the same magnitude of SFA- and MUFA-induced sparing of LC-PUFA is not observed in all circumstances and seems to vary according to fish species (e.g., White Seabass were able to maintain tissue fatty acid profile equivalent to control, but Florida Pompano were not able to maintain tissue profile), diets rich in SFAs and/or MUFAs still outperformed the other

experimental diets (rich in C₁₈ PUFAs) in terms of reducing diet-related losses of fillet LC-PUFA levels and preserving the overall fatty acid profile.

In recent years, n-3 LC-PUFAs have been highlighted as human health-promoting fatty acids mainly due to the anti-inflammatory actions of the signaling molecules (i.e., eicosanoids) derived from EPA and the various physiological and physical attributes of DHA. The value of n-3 fatty acids is most often described in comparison to the less beneficial, pro-inflammatory n-6 fatty acids. The Western diet is widely considered to contain far too much n-6 fatty acid content and too little n-3 fatty acid content. Consumers are urged to increase their consumption of n-3 fatty acid-rich foods (specifically seafood) in an attempt to balance out consumption of foods with high n-6 fatty acid levels. Thus, to maintain product quality and value, it is important that farmed seafood be as good a source of valuable n-3 fatty acids as wild fish, while containing as little n-6 fatty acid content as possible. In this context, SFA- and MUFA-rich alternative lipids offer dual advantage over C₁₈ PUFA-rich lipids: SFA- and MUFA-rich lipids minimize the loss of n-3 LC-PUFAs from farmed fish fillets and contain few n-6 fatty acids that would otherwise become enriched in the fillets. Although marine finfish accept a variety of alternative lipids, those rich in SFAs and MUFAs seems advantageous for feed formulations in terms of limiting tissue fatty acid profile distortion, enhancing LC-PUFA bioavailability, and in some cases, reducing LC-PUFAs requirements.

In terms of tissue total lipid content, it appears that effect of dietary lipid source on tissue lipid content may be species-specific and a function of the fatty acid composition of alternative lipids used. For example, although no statistically significant differences in total lipid content of fillet and liver tissues were observed in Florida Pompano, numeric differences appeared to suggest somewhat elevated lipid content in those tissues of fish fed the beef tallow-based feeds (low C₁₈ PUFA, high SFA/MUFA content), and reduced lipid content in tissues of those fed SFA-rich soybean oil (low C₁₈ PUFA/MUFA, high SFA content) or poultry fat (similar C₁₈ PUFA/SFA/MUFA content). Further, total lipid content of fillet and liver tissues of White Seabass was significantly reduced in fish fed SFA-rich soybean oil-based feeds (low C₁₈ PUFA/MUFA, high SFA content) compared to those fed fish oil (high LC-PUFA content) or C₁₈ PUFA-rich soybean oil-based feeds (high C₁₈

PUFA, low SFA/MUFA content). The value of farmed fish fillets is based on both fatty acid profile and total lipid content, thus further research to investigate the effects of SFA- and MUFA-rich feeds on tissue lipid content is warranted.

Conclusion

In summary, the present results provide further insights regarding fatty acid essentiality in marine carnivorous finfish, fatty acid composition of the alternative lipid source, degree of tissue fatty acid profile distortion, and also highlight the importance of assessing and providing LC-PUFAs on the basis of individual fatty acids. Not all LC-PUFAs are required in marine carnivorous fish feeds, with DHA and ARA being the primary drivers of fatty acid essentiality, whereas EPA is likely required in minor amounts. Further, DHA/EPA ratio has little-to-no effect on fish performance. The fatty acid composition of alternative lipid sources influences bioavailability of and requirements for LC-PUFAs. The requirements themselves seem to be more flexible than previously presumed. Finally, alternative lipids rich in SFAs and/or MUFAs are likely advantageous ingredients in aqua feeds in the context of preserving tissue fatty acid profile, and enhancing bioavailability of LC-PUFAs.

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Transcriptómica, la Nueva Puerta de Conocimiento para la Nutrición en Acuicultura: Actividad Enzimática Digestiva en Larvas de Crustáceos

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Resumen

El conocimiento del transcriptoma y su regulación es fundamental para la interpretación articulada de los diversos constituyentes moleculares que integran la red de respuesta génica de un individuo ante un evento inductor. La expresión o transcripción de los genes se ha implementado recientemente en estudios nutricionales de larvas. Las investigaciones que combinan los mecanismos reguladores de la expresión de genes con la actividad de las enzimas digestivas durante el desarrollo larval en peces son escasas y aún más en crustáceos.

En el presente documento se abordan algunos de los aspectos relevantes relacionados con el potencial de la transcriptómica en el estudio de la nutrición de larvas de crustáceos de interés comercial.

Palabras clave: Enzimas digestivas, Larvas crustáceos, Nutrición larvas, Transcriptómica

Introducción

El cultivo de crustáceos de importancia comercial comprende un amplio número de especies. En el mundo camarones langostas y cangrejos son cultivados mundialmente como fuente de alimento. La mayoría de estas especies producen un gran número de huevos, no obstante, la supervivencia y desarrollo exitoso de las larvas dependen de diversos factores, que representan grandes retos durante el proceso de producción. Las larvas de algunos crustáceos como los braquiópodos (*Artemia*), copépodos (*Tigriopus*) y Peneidos (*Penaeus*), eclosionan como nauplios, inicialmente lecitotróficas, pero en pocos días u horas se convierten en fitotróficas. En contraste la mayoría de decápodos eclosionan en un estado avanzado de zoea que usualmente es zootrófica desde su nacimiento (Jones *et al.* 1997b). A pesar de las diferencias entre ellas, es importante que los alimentos suministrados durante estos estadios provean los nutrientes necesarios para un adecuado desarrollo, el cual finalmente deberá reflejarse en mayores tasas de supervivencia y crecimiento.

La morfología del tracto digestivo durante el desarrollo ontogénico de las larvas es similar en la mayoría de decápodos. El desarrollo de los órganos secretores de enzimas digestivas como el hepatopáncreas son fundamentales para definir en el tipo de alimento que puede ser suministrado (De Silva y Anderson, 1995; Jones *et al.* 1997b; Houlihan *et al.* 2001). Estudios cuantitativos de las enzimas digestivas en grupos taxonómicos relativamente cercanos han revelado diferencias relacionadas con las fuentes de alimento disponibles. Altos niveles de proteasas y pocos de carbohidrasas son usuales en especies carnívoras y en sentido inverso para las herbívoras, mientras que los detritívoros y omnívoros se encuentran presumiblemente en una posición intermedia (Sather, 1969; Brun y Wojtowicz, 1976). Bajo este contexto, el análisis de la actividad enzimática en decápodos como langostas y cangrejos, ha demostrado ser una aproximación efectiva para entender la fisiología digestiva y determinar las características del alimento que debe ser suministrado (Hirche y Anger, 1987; Biesiot y Capuzzo, 1990; Lovett y Felder, 1990; Harms *et al.* 1991; Kamarudin *et al.* 1994; Saborowski *et al.* 2006; Rotllant *et al.* 2008; Andrés *et al.* 2010).

La expresión o transcripción de los genes se ha implementado recientemente en estudios nutricionales de larvas (Rodríguez *et al.* 2012). Las investigaciones que combinan los mecanismos reguladores de la expresión de genes con la actividad de las enzimas digestivas

durante el desarrollo larval son escasas en peces y aún más en crustáceos. Los pocos estudios desarrollados han permitido conocer de manera preliminar el comportamiento de las principales enzimas digestivas, relacionando algunas proteasas, lipasas, amilasas y enterasas con su expresión génica en algunas especies de camarones y de cangrejos adultos; mientras que en larvas solo hay estudios en camarones de los géneros *Penaeus* y *Litopenaeus*. Dichas investigaciones han estudiado las respuestas de las enzimas durante el desarrollo larval frente a diferentes dietas (Hirche y Anger, 1987; Harms *et al.* 1994; Klein *et al.* 1998; Sellos y van Wormhoudt, 1999; Muhlia-Almazán y García-Carreño, 2003; Van Wormhoudt y Sellos, 2003, Saborowski *et al.* 2006; Zhao *et al.* 2007; Rotllant *et al.* 2008; Andrés *et al.* 2010; Rivera-Perez *et al.* 2011).

El entendimiento del transcriptoma representa un gran potencial por descubrir en el campo de la nutrición, y al integrarse con otras ciencias moleculares como la proteómica, metabolómica y bioinformática, puede facilitar el descubrimiento de proteínas claves que regulan las rutas metabólicas importantes (Castellanos *et al.* 2004; Rodrigues *et al.* 2012), en especial en aquellas relacionadas con la nutrición de los organismos acuáticos de interés comercial.

Alimentación y actividad enzimática

La alimentación tiene efectos directos sobre la actividad enzimática digestiva, en ella se reflejan las adaptaciones a la disponibilidad y calidad del alimento suministrado. La nutrición es uno de los factores más importantes que influyen sobre las tasas de crecimiento y supervivencia (Felgenhauer *et al.* 1989; Le Vay *et al.* 2001). Algunos autores indican que con exceso de alimento, como ocurre al cultivar las larvas de crustáceos, los niveles de las enzimas digestivas se ven reducidos, ya que los requerimientos energéticos se alcanzan sin necesidad de realizar un proceso digestivo altamente eficaz, este modelo implicaría entonces que con poca disponibilidad de alimento o con baja disponibilidad de ciertos nutrientes que estén por encima del nivel de desnutrición, se presentarían altos contenidos enzimáticos. Esto podría explicar porque las larvas de herbívoros y omnívoros en peneidos como protozoas o mysis presentan mayor contenido de proteasas en comparación con las

carnívoras en carídeos u homaridos (Harris *et al.* 1986; Harms *et al.* 1991; Jones *et al.* 1997b).

El hecho de que la mayoría de células epiteliales del tracto digestivo en larvas de libre nado sean absorbentes, indican que la secreción de enzimas digestivas puede ser limitada en estos estadios tempranos. Por este motivo se presenta una relación directa entre la presencia/ausencia de alimento y su composición, con la actividad y con la concentración de enzimas digestivas. Bajo este contexto el análisis de la actividad enzimática digestiva es una herramienta que permite entender la fisiología digestiva y determinar las características nutricionales de un organismo (De Silva y Anderson, 1995; Andrés *et al.* 2010; Mireia *et al.* 2010). Los estudios de nutrición de las larvas de los crustáceos con los métodos tradicionales, indican que ésta parece depender de pocas enzimas digestivas, las cuales presentan altos niveles de actividad, en comparación con los adultos (Jones *et al.* 1997a, 1997b). Las larvas omnívoras presentan grandes variaciones en los niveles de proteasas, las cuales dependen de la composición del alimento suministrado. Algunas larvas de peneidos y cangrejos, reducen sus niveles de proteasas cuando son alimentadas con zooplancton altamente proteico, en comparación con aquellas que se alimentan con una dieta baja en proteínas (Jones *et al.* 1993; Rodríguez *et al.* 1994). Otras especies de peneidos incrementan sus niveles de tripsina en los estadios herbívoros de protozoas, alcanzando un pico al acercarse la transición de mysis y declinando al volverse carnívoros (Lovett y Felder, 1990; Jones y Kamarundi, 1993).

Mecanismos regulatorios de la expresión de genes y la producción de enzimas en larvas de crustáceos

El estudio de la biología molecular ha abierto diversos campos de investigación, como resultado se han desprendido múltiples ramas de estudio, entre ellos la transcriptómica. En las células, la información genética cifrada en el ADN y contenida en los genes se expresa a través de los mecanismos de transcripción y traducción, a partir del cual se producen moléculas de ARN mensajero (ARNm) y proteínas. El transcriptoma es el conjunto de todos los transcritos de ARN que se pueden generar en un momento y bajo una condición fisiológica determinada a partir del genoma de un organismo, estos están involucrados en

diversas funciones celulares, directamente como moléculas biológicas activas de ARN, o indirectamente a través de las proteínas que codifican los ARNm (Bolívar-Zapata, 2007; Soto & López, 2012).

Cualquier organismo marino en los estadios larvales experimenta cambios importantes a nivel celular y morfológico. Durante el periodo larval el desarrollo del tracto digestivo combina estos dos aspectos, en este proceso los mecanismos reguladores se presentan en diferentes niveles, y parecen estar genéticamente conectados, afectando los procesos de transcripción y traducción (Zambombino-Infante y Cahu, 2001; Muhlia-Almazán y García-Carreño, 2003). La cantidad de moléculas producidas de determinado ARNm depende de la función que este tenga en un proceso celular específico; así, cuando se requiere dar respuesta a una condición determinada en la cual un gen tiene una participación importante, más moléculas de este transcrito se producen; de manera similar, bajo circunstancias particulares hay genes que permanecen apagados, pero bajo la ocurrencia de un estímulo particular, se expresan y se inicia la transcripción correspondiente.

Los mecanismos involucrados con el lugar, el cómo y el cuándo se genera un transcrito, son fundamentales para el entendimiento de la actividad biológica de un gen; más aún, los niveles de ARNm pueden dar una visión clara de patrones de expresión, pero además permiten realizar cuantificaciones altamente correlacionadas entre la abundancia de ARNm y la abundancia de proteínas (Lockhart & Winzeler, 2000).

La interpretación de la expresión génica requiere disponer de información del genoma del ADN y los ARN expresados, los cuales se han desarrollado en muy pocas especies de importancia acuícola, los más estudiados son los peces (Rodrigues *et al.* 2012). El control genético en la síntesis de enzimas puede conducir a: 1) Cambios en la cantidad de una enzima producida, 2) Expresión de diferentes enzimas dentro de una célula, o 3) Cambios en la abundancia relativa de enzimas que catalizan una reacción. La mayoría de los organismos sintetizan diferentes enzimas llamadas isoformas o isoenzimas que catalizan una sola reacción y son codificadas por diferentes genes (Hochachka y Somero, 1984; Resch-Sedlmeier y Sedlmeier, 1999; Forne *et al.* 2010).

En la década de los noventa se desarrollaron diferentes métodos para la detección y cuantificación de ARNm a partir de muestras biológicas como el *Northern blotting*, RT-PCR (del Inglés *Reverse Transcription Polymerase Chain Reaction*), microarreglos de

ADNc (ADN complementario obtenido por transcriptasa inversa a partir de ARNm), los cDNA-AFLP (del inglés *Amplified Fragment Length Polymorphism*) y el análisis serial de expresión de genes SAGE (del inglés *Serial Analysis of Gene Expression*), entre otras técnicas. Estos procedimientos permitieron generar conocimiento en transcriptómica, al estudiar la expresión de genes bajo estímulos particulares, también fue posible determinar cambios en los patrones de expresión génica en diferentes tratamientos y conocer las cinéticas de expresión (Shalon *et al.* 1996; Schena *et al.* 1998; Ferrer *et al.* 2004; Meyers *et al.* 2004; Valdés *et al.* 2013). Sin embargo, estas técnicas enmascaran la detección de transcritos de baja abundancia, por tener baja cobertura (Ward *et al.* 2012).

En estudios relacionados en larvas de peces marinos se ha descrito el desarrollo del sistema digestivo y la formación de órganos mediante histología, detallando la dinámica de las principales enzimas digestivas y la expresión de genes en diferentes tipos de tripsinas, pepsinas y quimiotripsinas (proteasas), amilasas, leucina y quitinasas (carbohidrasas) y algunas lipasas y enterasas. Los experimentos se han enfocado a etapas finales larvales o al inicio de la juvenil, para investigar la fase de destete en especies como corvina (*Larimichthys crocea*) mero (*Labrus bergylta*), lubina (*Lates calcarifer*), dorada (*Sparus aurata*) bacalao (*Gadus morhua*), lenguado (*Solea solea*) y pez globo (*Sphoeroides annulatus*); en general los estudios indican que existen cambios a lo largo del desarrollo larval, los cuales ocurren como respuesta a los tipos de dietas suministradas o bien a los períodos de ayuno (Zambombino-Infante y Cahu, 2001; García-Gasca *et al.* 2006; Kortner *et al.* 2011; Parma *et al.* 2013; Srichanun *et al.* 2013; Truls-Wergeland *et al.* 2013; Cai *et al.* 2015; Mata-Sotres *et al.* 2016; Canada *et al.* 2017).

En crustáceos se han reportado muy pocos trabajos relacionados con el tema. Estudios con larvas de *Litopenaeus schmitti* bajo un solo esquema de alimentación se realizaron para determinar las variaciones en la concentración de las endopeptidasas tripsina y quimotripsina y su relación con concentraciones de ARN y ADN (Chomczynski y Sacchi, 1987; Chomczynski, 1993; Green y Sambrook, 2012), encontrando que ambos contenidos se reducen desde el huevo a protozoa III, incrementando paulatinamente hasta poslarva IV. La variación en la actividad enzimática también se relaciona con diferentes estrategias para el uso de la energía a lo largo del desarrollo larvario. La elevada actividad enzimática combinada con un incremento del ARN/ADN en los estadios de protozoa, denotan que en

este periodo es necesario suministrar una dieta con alto contenido energético, que permita al organismo acumular reservas para el posterior crecimiento (Lemos *et al.* 2002).

Actualmente los avances en las técnicas de secuenciación del ADN, a través de tecnologías de nueva generación, NGS (del inglés *Next Generation Sequencing*), permiten caracterizar y analizar toda la expresión génica de una célula o tejido, aun sin ninguna información genómica previa, esto es posible a través de la implementación de la secuenciación de ADNc, o más recientemente de la secuenciación directa de ARN, tecnología conocida como RNA-seq, la cual cuenta con plataformas abiertas dirigidas a secuenciaciones directas de bibliotecas de ácidos nucleicos con un alto rendimiento. Las plataformas más usadas actualmente son de la compañía *Illumina* siendo la maquina HiSeq la preferida por su rendimiento y costos. Estas herramientas han cambiado la forma de analizar y comprender los transcriptomas. La información generada, una vez integrada e interpretada permite vislumbrar procesos biológicos y mecanismos de co-expresión. Independientemente de la plataforma escogida para la secuenciación los procesos comparten varias etapas comunes (Wang *et al.* 2009; Garber *et al.* 2011; Sánchez-Pla *et al.* 2012; Soto y López, 2012; Ward *et al.* 2012; Valdés *et al.* 2013; Capobianco, 2014) entre las que destacan:

1. Preparación de las bibliotecas que involucran la fragmentación de las moléculas de ARN, síntesis de ADNc y ligamiento de adaptadores específicos a ambos extremos de la cadena.
2. Amplificación por clonación de cada molde, ya que la mayoría de sistemas de imágenes no han sido diseñados para detectar eventos fluorescentes individuales.
3. Unión de los ADN moldes amplificados a un soporte sólido, ya sea en una celda de flujo o una cámara de reacción.
4. Flujo continuo y sincronizado, así como lavado de reactivos para la extensión de las hebras de ADN, mientras que las señales son obtenidas por el sistema de detección.
5. Luego de obtenida la señal, la imagen cruda de datos conseguida debe convertirse en lecturas cortas (secuencia de nucleótidos generada por los ADN modelos) por un proceso conocido en inglés como *base-calling*, dependiendo de la plataforma la extensión o síntesis de ADN puede obtenerse enzimáticamente por polimerización.

Las aplicaciones de este tipo de metodologías en el campo de los alimentos y la nutrición son relativamente nuevas, en comparación a sus aplicaciones a las ciencias básicas, y como ya se comentó las investigaciones enfocadas a la nutrición de larvas de crustáceos, son incipientes. Solo recientemente se reportó un estudio en larvas de *Litopenaeus vannamei* durante cinco estadios larvales: embriones, nauplios, zoeas, mysis y poslarvas aplicando la tecnología RNA-seq, secuenciando las librerías obtenidas usando Illumina HiSeq™ 2000 (Wei *et al.* 2014). Las lecturas de cada estadio se ensamblaron en unigenes, los cuales se obtienen mediante programas especializados que realizan el agrupamiento de secuencias relacionadas, posteriormente los unigenes obtenidos de los cinco estadios fueron unidos, analizados y mapeados con ayuda de programas bioinformáticos especializados, realizando alineamientos e identificando los genes por blast (Altschul *et al.* 1990; Pertea *et al.* 2003; Grabherr *et al.* 2011). El agrupamiento de genes (*gene clustering*), permite asociar aquellos que tienen perfiles de expresión similares, esto es útil para descubrir genes que se co-regulan y que pueden participar en procesos biológicos similares, o bien posibilita la reducción en la dimensionalidad de los datos (Valdés *et al.* 2013).

Continuando con el estudio de Wei *et al.* (2014), entre los 66815 unigenes que se obtuvieron, 296 correspondieron a 16 tipos de enzimas digestivas diferentes, incluyendo cinco carbohidrasas, siete peptidasas y cuatro lipasas las cuales han sido descritas con anterioridad para camarones adultos o juveniles de la misma especie, así como en *L. vannamei* y *P. monodon* (Klein *et al.* 1998; Sellos y van Wormhoudt, 1999; Le Chevalier *et al.* 2000; Muhlia-Almazán *et al.* 2003; Van Wormhoudt y Sellos, 2003; Zhao *et al.* 2007; Huang *et al.* 2010; Proespraiwong *et al.* 2010; Rivera-Perez *et al.* 2011).

Con este tipo de estudios se elucidan los caminos metabólicos relacionados con la ingestión y absorción del alimento, pudiendo clasificar cientos de genes dentro de estas vías, no solo las enzimas digestivas. Esto es posible mediante un proceso de mapeo de genes con las bases disponibles en la web, conocido como Kyoto Encyclopedia of Genes and Genomes - KEGG (Tabla 1).

Una forma clara de visualizar los patrones de actividad de las enzimas digestivas (α -amilasa, es a través mapas térmicos, en los cuales se observa como los unigenes expresados incrementan gradualmente a lo largo del desarrollo larval. Wei *et al.* (2014) muestran en su

estudio un ejemplo del potencial de éstas herramientas. Este tipo de estudios pueden revelar la diversidad y la dinámica de las enzimas digestivas durante el desarrollo larval, además proveen un soporte para entender mejor los cambios fisiológicos durante la transición de la dieta de acuerdo a su desarrollo.

Tabla 1. Rutas metabólicas y conteo de unigenes KEGG relacionados con la ingestión y absorción (Tomado de: Wei *et al.* 2014).

Rutas metabólicas	Conteo de unigenes
Secreciones salivales	572
Digestión y absorción de proteínas	519
Secreciones pancreáticas	443
Metabolismo de azúcares de nucleótidos y aminoazúcares	214
Metabolismo de glicerolípidos	170
Metabolismo de almidón y sacarosa	143
Digestión y absorción de lípidos	114
Metabolismo de manosa y fructosa	114
Metabolismo de galactosa	106
Metabolismo de ácidos grasos	99

El objetivo clave de la transcriptómica es preciso y consiste en caracterizar todo el transcriptoma abarcando cualquier tipo de transcripción incluyendo el ARNm y ARN no codificantes, determinar la estructura de la transcripción de genes, por ejemplo, sitios de inicio 5' y finales 3' y variantes de empalme y la cuantificación de los niveles de expresión diferencial de cada transcripción bajo diferentes condiciones (Valdés *et al.* 2013).

Conclusión

Este tipo de investigaciones trazan el camino inicial hacia el entendimiento profundo de la nutrición con aplicación en el cultivo de crustáceos, usando como herramienta básica la biología molecular y la expresión de los genes ante los factores medioambientales, o ante las variaciones de las dietas. Los procesos de transcripción acoplados a otras ramas

moleculares como la proteómica, metabolómica y bioinformática, podrán en conjunto ayudar a entender y plantear estrategias nutricionales efectivas, las cuales contribuirán a mejorar los índices de supervivencia y de crecimiento en larvas de crustáceos de interés comercial.

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Uso De Microorganismos Inmovilizados en Cultivos Camaronícolas y su Efecto en la Respuesta Productiva, Condición Fisiológica y Microbioma Intestinal

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Resumen

Los microorganismos juegan roles importantes en el cultivo de especies acuáticas, incluyendo el mejoramiento de la respuesta productiva, condición fisiológica e inmune y calidad del agua en los sistemas de producción y sus efluentes. Nuestro grupo de trabajo se ha enfocado, además, en aspectos tales como su efecto en la modificación del microbioma intestinal y en la expresión de genes de importancia para propósitos acuaculturales. El documento resume los resultados de nuestras investigaciones sobre caracterización, manejo y uso de microorganismos inmovilizados incluyendo: evaluación de materiales para la formación de biopelículas, efecto de biopelículas y bioflóculos en la respuesta productiva del camarón, en la expresión de genes relacionados con el sistema inmune y en la modificación del microbioma intestinal del camarón blanco. Entre los resultados sobresalientes, se encontró: 1) que superficies sintéticas como la malla-sombra son más eficientes en la formación y estabilidad de las biopelículas; 2) que las biopelículas formadas en este material tienen una composición bioquímica adecuada para la nutrición del camarón; 3) que el consumo de biopelículas foto-autotróficas o heterotróficas, puede mejorar el crecimiento hasta en un 20%, la supervivencia alrededor de un 15 % y disminuir el FCA hasta en 0.5 unidades; 4) que la inclusión de biopelículas en sistemas de cultivo de camarón y tilapia reduce significativamente las concentraciones de nitrógeno amoniacal; 5) que el microbioma intestinal del camarón se ve notablemente modificado por el consumo de microorganismos asociados a biopelículas y 6) que genes relacionados con el sistema inmune se sobre-expresan en camarones que consumen microorganismos asociados a biopelículas.

Palabras claves: Microorganismos inmovilizados; biopelículas, bioflóculos, camarón, tilapia, microbioma intestinal, expresión de genes.

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Introducción

Ante la seria problemática que representa el alimento formulado para la acuicultura en términos de disponibilidad, costo e impacto ambiental, importantes y diversas investigaciones se han enfocado en la búsqueda de alternativas alimentarias más ecoeficientes y económicas. Los microorganismos han sido utilizados desde hace ya algunos años como un complemento alimenticio adecuado para organismos acuícolas como peces y camarones (Martínez-Córdova *et al.* 2015). La manera en que los camarones pueden aprovechar mejor a los microorganismos, es cuando se encuentran inmovilizados en superficies fijas (biopelículas) o flotantes (bioflóculos). La tecnología de bioflóculos se inició hace poco más de dos décadas (Avnimelech, 1995), pero actualmente ha sido optimizada y ampliamente utilizada para el cultivo de varias especies, pero mayormente en tilapia (Miranda-Baeza *et al.* 2017), y camarones peneidos (Emerenciano *et al.* 2017). Las biopelículas se han empleado en menor medida y de manera más artesanal como por ejemplo el perifiton adherido a superficies de palos, bambú, etcétera (Anand *et al.* 2015). Como alternativa tecnológicamente más desarrollada de biopelículas, se tiene el uso de los llamados aquamats, los cuales son superficies artificiales teóricamente diseñadas para la adhesión de organismos específicos, pero con un costo que en términos generales no justifica su uso, ya que otros materiales menos costosos pueden dar un resultado similar (Audelo-Naranjo *et al.*, 2015).

Los beneficios del uso de los microorganismos inmovilizados, no es solamente su aportación como alimento complementario, sino que además contribuyen al mejoramiento de la calidad del agua del sistema de cultivo (Miao *et al.*, 2017), en algunos casos actúan como probióticos, inmunoestimulantes o inmunomoduladores (Kethi *et al.*, 2017); adicionalmente pueden contribuir a mejorar la función enzimática digestiva de los organismos que los consumen por la aportación de enzimas exógenas (Anand *et al.*, 2017).

Desde hace ya algunos años, la Universidad de Sonora, en colaboración con otras instituciones nacionales como el CIAD, la UES y el CIBNOR, se ha enfocado a la caracterización, uso y manejo de microorganismos y su contribución en el cultivo de camarón

y tilapia en términos de respuesta productiva y condición fisiológica e inmune (Becerra-Dórame *et al.* 2012, 2014; Miranda-Baeza *et al.* 2016). Más recientemente, las investigaciones se han enfocado en estudiar el efecto que estos microorganismos tienen sobre la microbiota intestinal de los camarones, así como en la expresión de algunos genes relacionados sobre todo con el sistema inmune y la actividad enzimática digestiva. Hay evidencias documentales que sustentan la hipótesis de que la ingestión de microorganismos puede modificar el microbioma intestinal de diversos organismos incluyendo aquellos que son objeto de acuicultura (Vargas-Albores *et al.*, 2017; Garibay-Valdez, 2017 *En prensa*). Esta modificación puede tener efectos importantes en el desarrollo de los organismos en aspectos tales como crecimiento, supervivencia, FCA, eficiencia de asimilación proteica, entre otras (Martinez-Cordova *et al.* 2015). Igualmente está documentado que bacterias y otros microorganismos, al ser ingeridos, tienen un efecto sobre la expresión de algunos genes relacionados con respuesta inmune y actividad enzimática (Xu *et al.*, 2013).

Algunos de los resultados más sobresalientes obtenidos por nuestras instituciones son presentados a continuación:

A. Evaluación de materiales naturales y sintéticos como superficies para la formación de biopelículas fotoautotróficas.

Se evaluaron tres materiales: madera, ixtle y malla-sombra plástica como superficies para la formación de biopelículas. Se utilizaron dos microalgas bentónicas: *Navícula incerta* y *Navícula* sp., ambas cultivadas en el laboratorio del DICTUS a partir de inóculos obtenidos del cepario del propio departamento. La fase experimental, se realizó al interior, con luz fluorescente a una irradiancia de 280 $\mu\text{mol}/\text{m}^2/\text{seg}$. Se utilizaron acuarios de plástico de 40 L en los que se colocaron los materiales a evaluar por triplicado. Después de cinco días se desprendió el material adherido a cada superficie de cada unidad experimental, re suspendiéndolo por agitación en 5 litros de agua. Se tomó una muestra de 300 mL, la cual se filtró en papel Whatman GFC. Por medio de la técnica de secado e ignición se estimó la materia seca y la materia orgánica. Se determinó además la composición químico proximal por los métodos convencionales (AOAC, 2010).

Tal como se muestra en la Figura 1, las mayores cantidades de materia seca (MS) y materia orgánica (MO), se obtuvieron con la microalga *Navícula incerta*, independientemente del

material utilizado. Respecto a las superficies evaluadas, la mayor cantidad de MS y MO, se encontraron en el material de madera, sin embargo, mucha de esta materia provino de la propia madera, por lo que no se pudo constatar la cantidad real correspondiente a la biopelícula. La segunda mayor cantidad de MS y MO, se recuperó del material de tela de ixtle, aunque igualmente, mucho del propio material se desprendió y se contabilizó como biopelícula, además de que otra cantidad no se pudo recuperar debido a la naturaleza del material. La malla-sombra de plástico, aunque concentró la menor cantidad de MS y MO, mostró la mejor estabilidad y por lo tanto fue el material elegido.

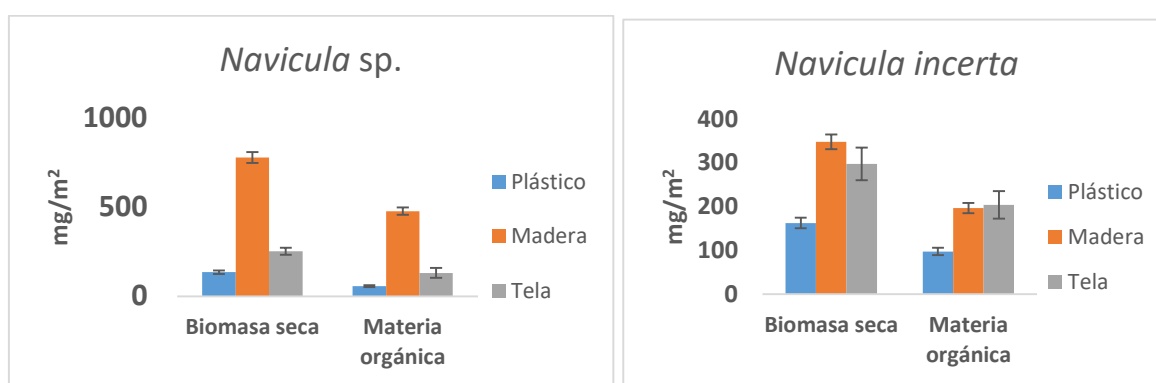


Figura 1. Materia seca y materia orgánica en biopelículas de *Navicula sp.* (a) y *Navicula incerta*, en superficies de malla-sombra plástica, madera y tela.

Respecto a la composición químico-proximal de las biopelículas, la mayor concentración de proteína se obtuvo en madera con *Navicula sp.*, muy similar al valor obtenido en malla-sombra con *N. incerta*. La concentración de carbohidratos fue mayor en la superficie de madera para las dos especies y el valor más bajo correspondió a la superficie de ixtle para *N. incerta*. Los lípidos presentaron concentraciones más altas en ixtle para *Navicula sp.* y más bajos en madera para *N. incerta*.

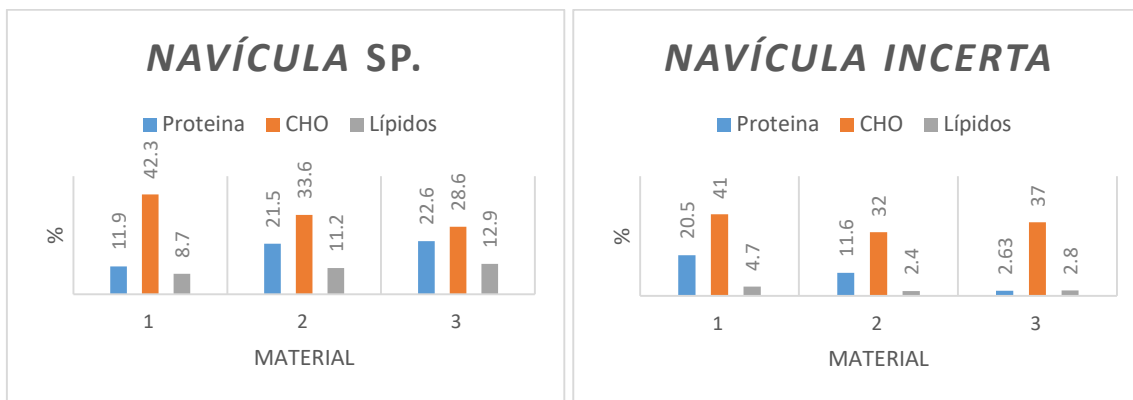


Figura 2. Composición químico-proximal (%) de las biopelículas formadas con *Navícula sp.* y *Navícula incerta* en superficies de plástico (1), madera (2) y tela de ixtle (3)

B. Efecto del consumo de biopelículas fotoautotróficas y heterotróficas en la respuesta productiva y calidad del agua del sistema.

Se llevó a cabo un experimento al exterior, en donde se probaron tres tipos de biopelículas: 1. Foto-autotróficas; 2. Heterotróficas inespecíficas (consorcio marino) y 3. Heterotróficas con un probiótico comercial específico.

Las biopelículas tipo 1, se produjeron en tinas de 200 L, al exterior y cubiertas con plástico transparente, en donde se inoculó una microalga bentónica que fue promovida con fertilizante agrícola. Se introdujeron cortinas de malla-sombra plástica. Se mantuvo una aireación moderadamente fuerte y constante hasta la formación de las biopelículas.

Las biopelículas tipo 2 y 3, se produjeron en tinas de 200 L con aireación constante y ausencia de luz. Se enriquecieron con melaza y fertilizante agrícola en cantidades tales que se obtuviera una concentración de carbono orgánico de ± 50 mg/L y una relación C:N de 20:1. Una vez que las biopelículas estuvieron formadas (al menos 1 mg de peso seco/cm²), se retiraron para ser probadas en acuarios de 60 L, en los que previamente se introdujeron 12 camarones con un peso promedio de 7 g. Cuando las biopelículas fueron consumidas casi totalmente, se reemplazaron por otras nuevas. Los camarones fueron además alimentados con un formulado comercial con 30 % de proteína, ajustando la ración de acuerdo al consumo aparente.

Diariamente se midieron los parámetros de calidad del agua: temperatura, salinidad, oxígeno disuelto, NAT y fosfatos, utilizando una sonda multiparamétrica YSI 6600 para los

parámetros fisicoquímicos y un espectrofotómetro programable HANNA para los nutrientes. Semanalmente se hicieron biometrías para conocer el peso promedio y la supervivencia, con estos datos se estimó el FCA.

Los resultados mostraron que las concentraciones de NAT y P-PO4 fueron significativamente menores en los tratamientos que incluyeron biopelículas (Figuras 3 y 4). El crecimiento, la supervivencia, la biomasa final y el FCA fueron mejores en los tratamientos que recibieron biopelículas tanto autótroficas como heterotróficas (Figura 5).

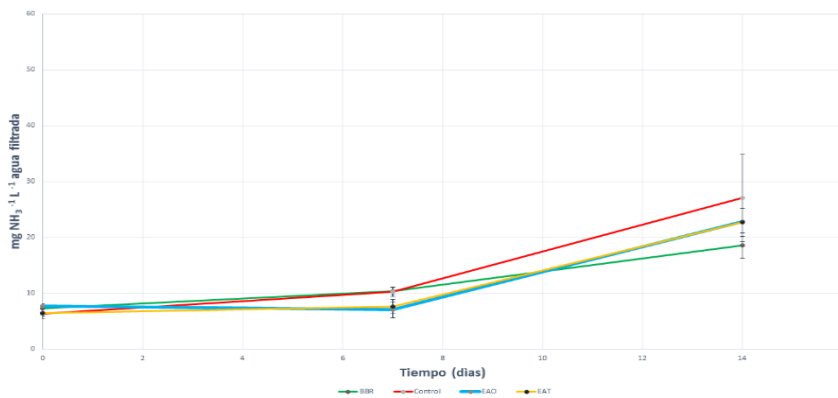


Figura 3. Concentración de nitrógeno amoniacal en el control (rojo) y con biopelículas.

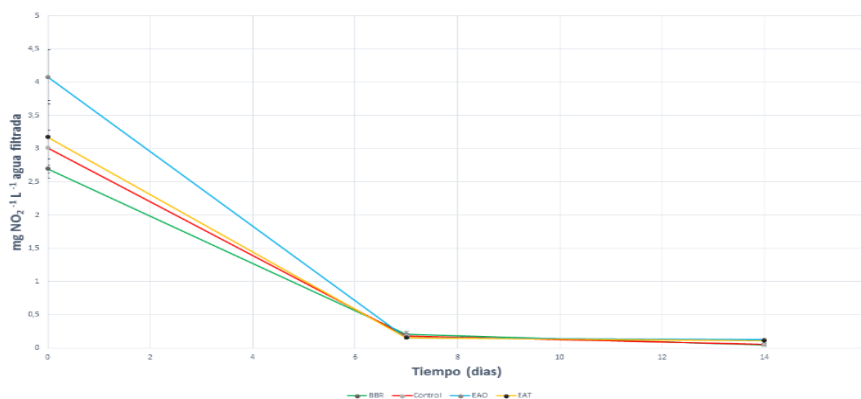


Figura 4. Concentración de fosfatos en el control (rojo) y con biopelículas.

La respuesta productiva del camarón mostró diferencias significativas entre los tratamientos. La supervivencia fue mayor en los tratamientos con biopelículas de organismos heterotróficos inoculados con el consorcio marino y con organismos foto-autotróficos

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(microalgas), mientras que el control mostró la supervivencia más baja después de siete semanas (Figura 5).

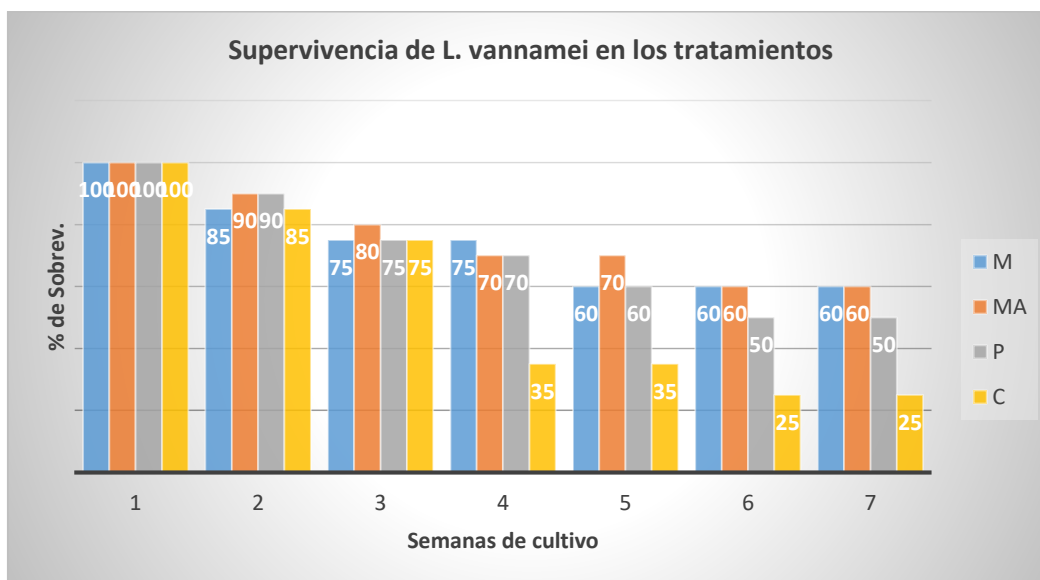


Figura 5. Supervivencia del camarón en el control (C) y en los tratamientos con biopelículas inoculadas con un consorcio marino (M), con un probiótico comercial (P) y con microalgas (MA)

El crecimiento fue mayor en el tratamiento donde se usaron las biopelículas de organismos heterótrofos marinos, mientras que el control presentó los menores valores (Figura 6).

El factor de conversión alimenticia fue más bajo en el tratamiento con biopelículas del consorcio marino y más alto en el control (Figura 7).

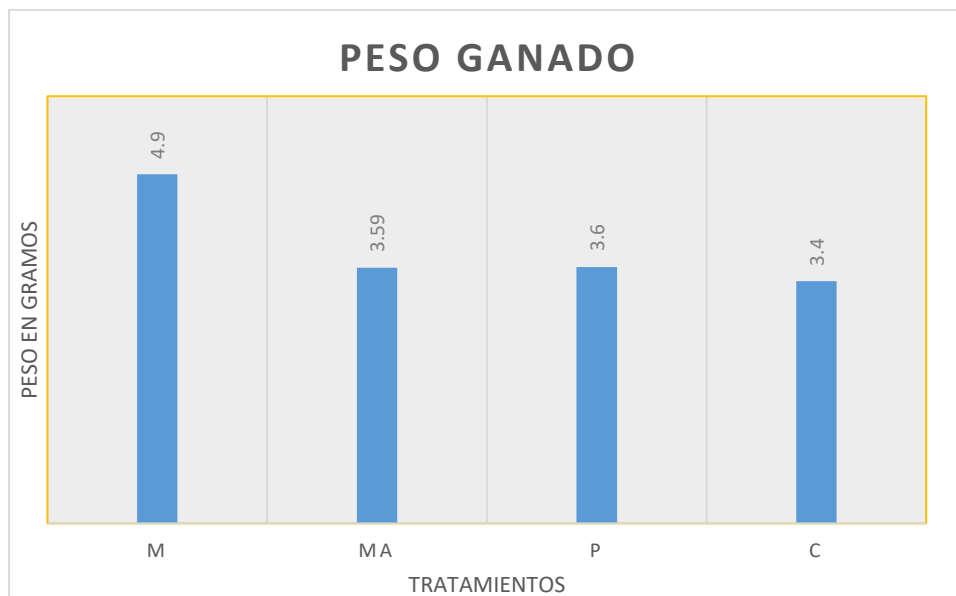


Figura 6. Peso ganado (g) de *L. vannamei* en los tratamientos con biopelículas heterotróficas de un consorcio marino (M); heterotróficas de un probiótico comercial (P), fotoautotrófica de microalgas (MA) y Control (C).

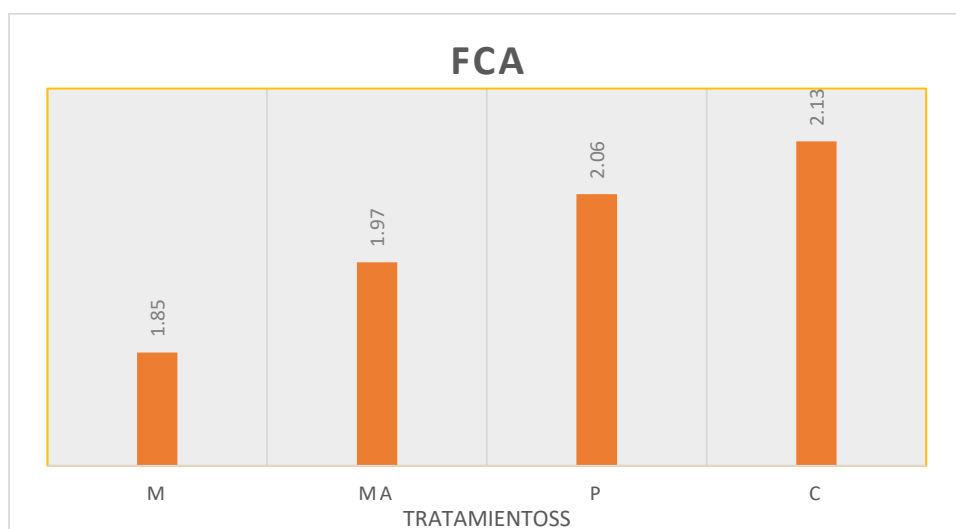


Figura 7. Factor de conversión alimenticia de *L. vannamei* en los tratamientos con biopelículas heterotróficas de un consorcio marino (M); heterotróficas de un probiótico comercial (P), fotoautotrófica de microalgas (MA) y Control (C).

C. *Diversidad microbiana intestinal del camarón alimentado con y sin biopelículas foto-autotróficas.*

Se estudió el cambio en el microbioma de camarones después de ser alimentados con biopelículas basadas en microalgas bentónicas en comparación con camarones que no recibieron esta fuente de alimentación. El estudio se llevó a cabo al exterior en tinas de plástico de 60 L de capacidad, cubiertas con plástico transparente, aireación constante y sin recambio de agua (reposición por evaporación). Los camarones del tratamiento control, fueron alimentados con una dieta comercial, ajustando la ración diaria de acuerdo al consumo aparente; los organismos de los tratamientos fueron además alimentados con biopelículas formadas por microalgas (*Navicula incerta*), producida aparte. Una vez que los organismos consumían toda la biopelícula, ésta era cambiada por una nueva durante todo el bioensayo. Se tomaron muestras de intestino de los organismos del control y de los tratamientos a los días 0, 15 y 30. Se extrajo el intestino de tres camarones por acuario, los cuales fueron juntados, homogenizados y colocados en tubos de 1.5 mL. Las muestras de biopelículas se tomaron por medio de raspado de superficie de manera aleatoria (4x4 cm). Las muestras se agruparon por cada acuario y se colocaron en tubos estériles de 1.5 mL.

Para la extracción de ADN de ambos tipos de muestras se utilizó el kit de aislamiento Power Biofilm (MP BIO Laboratories, Solana Beach, CA, EUA). La extracción se realizó según los pasos indicados en el protocolo del kit.

La detección de taxones se llevó a cabo mediante secuenciación masiva del gen ribosomal 16S (ARNr), considerando las regiones variables 3 y 4. Se utilizó un secuenciador de siguiente generación Illumina MiSeq.

Los resultados mostraron que la diversidad bacteriana de las biopelículas es bastante amplia a pesar de que se induce solo la adición de microalgas (Figura 8).

Los géneros de bacterias mayormente encontrados se muestran en la Figura 9, en donde se observa que la estructura poblacional bacteriana se basa en cuatro filos principales (Proteobacteria, Bacteroidetes, Firmicutes y Actinobacteria), mientras que a nivel de género destaca *Vibrio*.

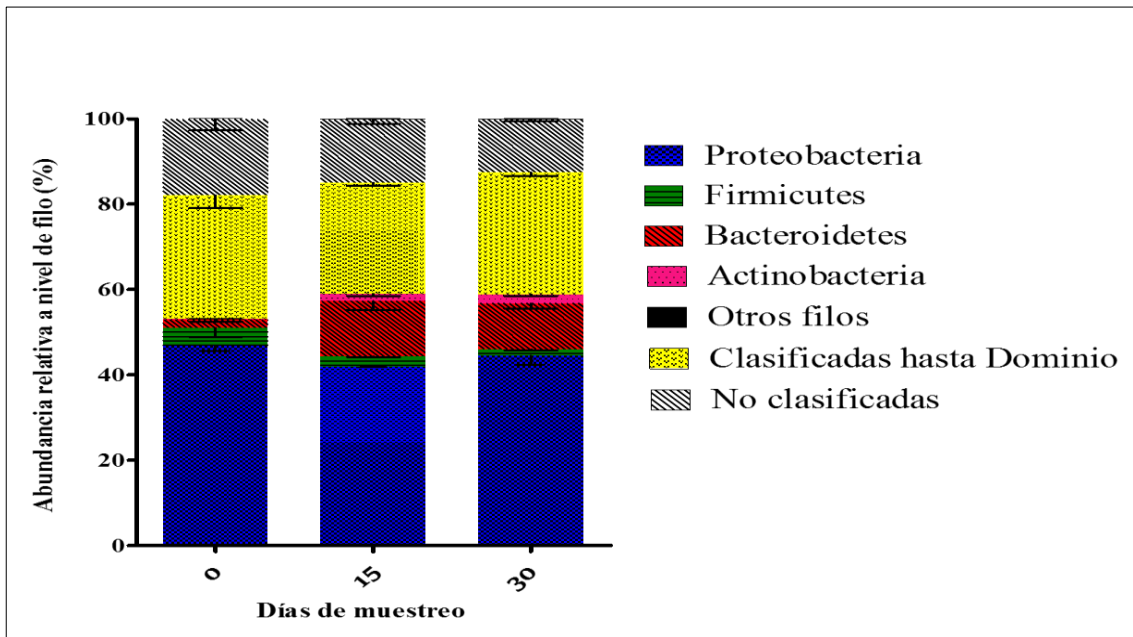


Figura 8. Grupos de bacterias mayormente encontrados en las biopelículas foto-autotróficas introducidas a un cultivo de camarón blanco.

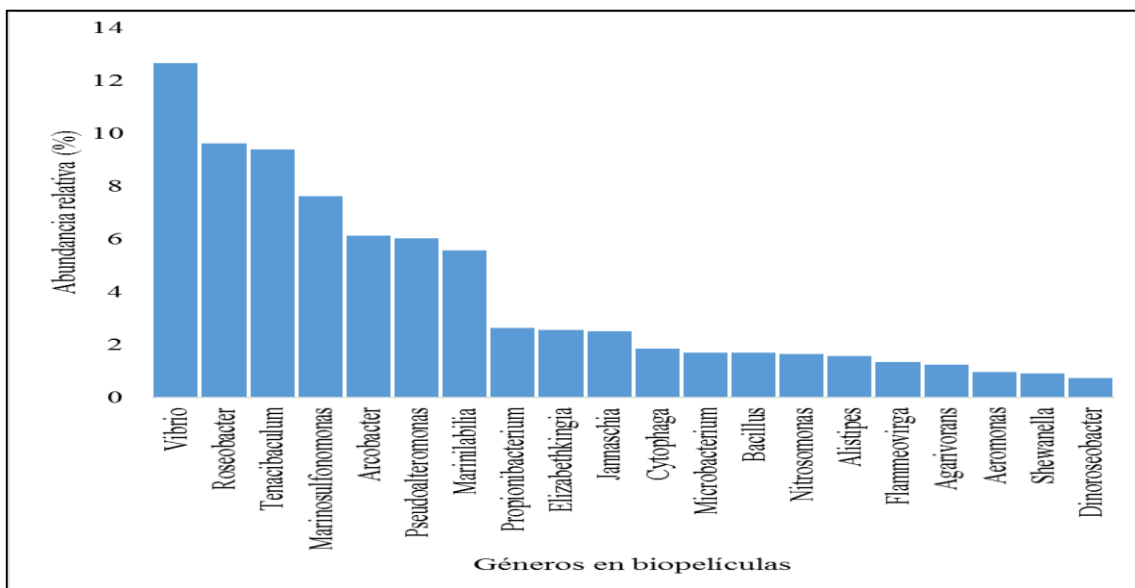


Figura 9. Géneros de bacterias mayormente encontrados en las biopelículas foto-autotróficas introducidas a un cultivo de camarón blanco.

La adición de biopelículas modificó la composición bacteriana intestinal de los camarones, aumentado la abundancia relativa de ciertas bacterias o favoreciendo su permanencia en el intestino del hospedero, y disminuyendo la abundancia de otras, tal como se muestra en la Figuras 11 y 12.

Los resultados de beta diversidad, mostraron que el consumo de biopelículas modificaba la microbiota intestinal del camarón, aunque se desconoce si estos cambios aportan nuevas funciones a la microbiota, por lo que se espera llevar a cabo estudios para inferir el potencial funcional de este tipo de consorcios y microbiota. Sin embargo, se vislumbra que el efecto positivo de las biopelículas sobre la supervivencia y crecimiento, podría deberse no solamente al aporte de nutrientes y mejoramiento de la calidad del agua, sino a un efecto a nivel fisiológico al tener incidencia sobre la microbiota intestinal, ya que esta se considera como un “órgano adicionado” a los organismos pertenecientes al reino animal, debido a las múltiples funciones que aporta (Clarke *et al.* 2014).

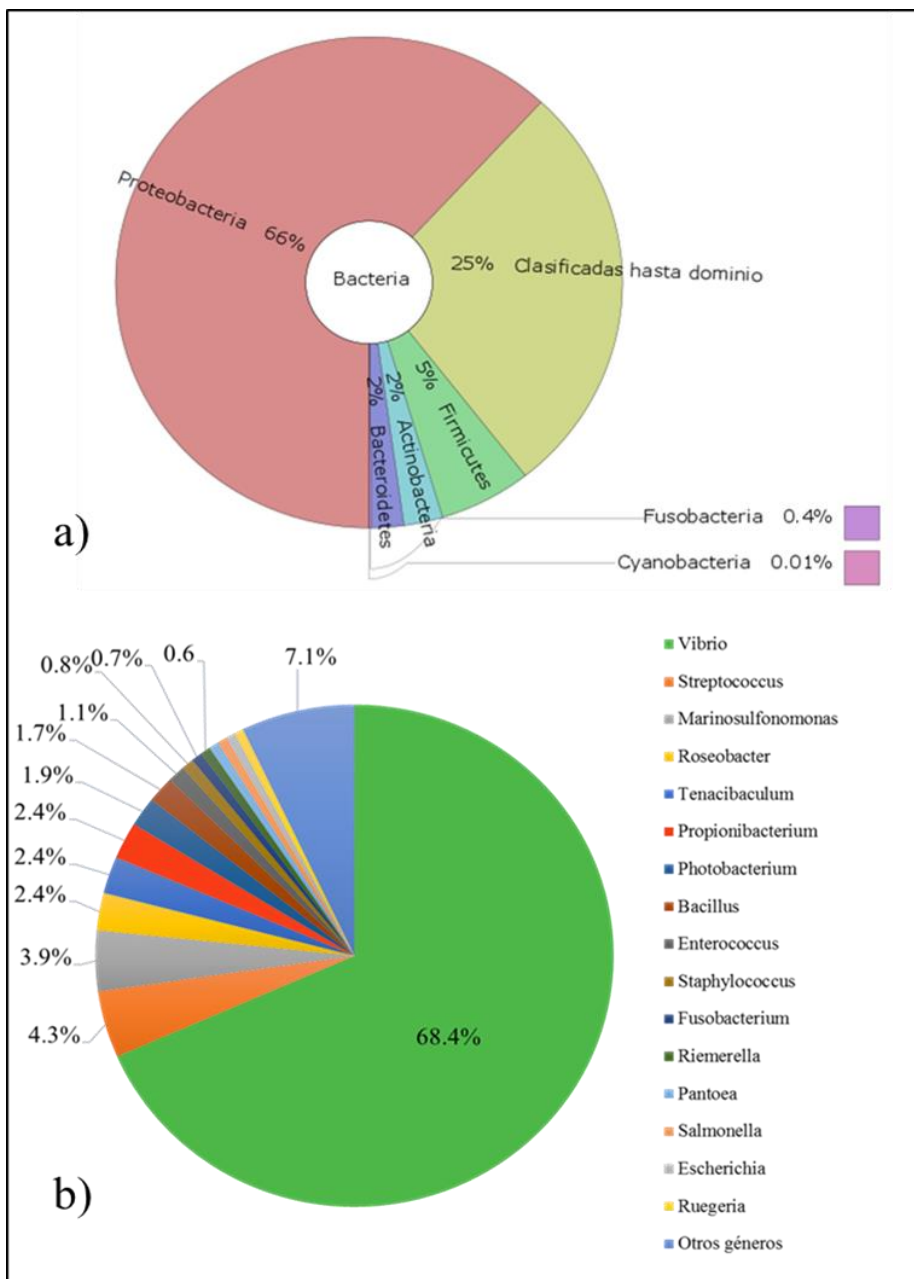


Figura 11. Abundancia relativa de la composición bacteriana intestinal de camarón blanco antes de la alimentación suplementada con biopelículas. a) A nivel de filo b) A nivel de género. Los géneros presentados son los que se encontraron con una proporción mayor a 0.5%

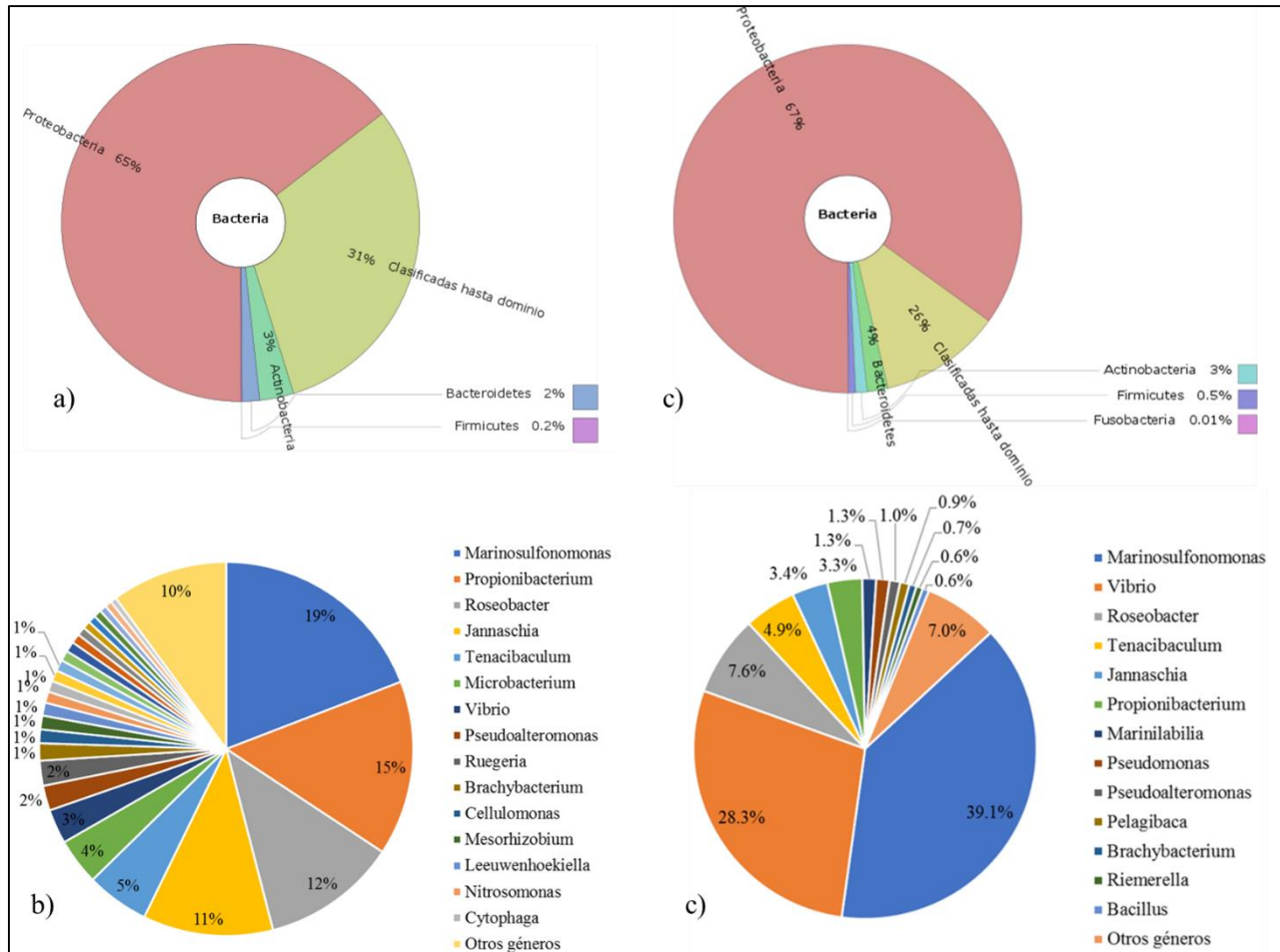


Figura 12. Abundancia relativa de la composición bacteriana intestinal de camarón blanco 15 días después de la alimentación suplementada con biopelículas. a) A nivel de filo en organismos control y b) A nivel de género en organismos control. c) A nivel de filo en organismos con biopelículas y d) A nivel de género en organismos con biopelículas. Los géneros presentados son los que se encontraron con una proporción mayor a 0.5%

D. Expresión de genes relacionados con el sistema inmune en camarones alimentados con bioflóculos.

Se llevó a cabo un estudio para evaluar la expresión de dos genes relacionados con el sistema inmune en camarones a los que se les suministraron bioflóculos como complemento alimenticio, en comparación con aquellos que no lo recibieron.

Dos grupos de camarones, asignados cada uno a 3 acuarios de 60 L con 12 organismos por acuario, fueron cultivados al exterior por 3 semanas. El primer grupo (C o Control), se alimentó con un formulado comercial con 30 % de PC. El otro grupo (T o tratamiento), se alimentó, además, con bioflóculos basados en organismos heterotróficos (consorcio marino inespecífico). Al final del estudio, a 3 camarones de cada acuario, se les extrajo hemolinfa, la cual fue analizada para evaluar la expresión de los genes BGPB y ProPO. Los camarones restantes fueron sometidos a un reto con *Vibrio parahaemolyticus* y virus de la mancha blanca, al final del cual se evaluaron nuevamente los mismos genes.

Para los análisis de expresión génica primero se extrajo el ARN de los hemocitos presentes en la hemolinfa por el método TRIzol®, del cual se cuantificó y analizó la integridad por espectrofotometría. A partir de las mediciones de cada muestra, todas se ajustaron a la misma concentración (4ng/μl). Seguidamente se realizó la retrotranscripción con el kit IScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) siguiendo las instrucciones del fabricante, quedando finalmente el ADNc a una concentración de 1 ng/μl. Los análisis por PCR en tiempo real se llevaron a cabo en un termociclador StepOne de Applied Biosystem (Thermo Scientific, EUA). Se analizaron los patrones de expresión de los genes profenoloxidasa (ProPO) y la Proteína beta de unión a glucanos (β-glucan binding protein (BGBP)). Como gen endógeno (housekeeping) se usó un fragmento del gen de la proteína ribosomal L8, el cual sirvió para normalizar los datos de cada uno de los dos genes mencionados anteriormente. Los cebadores empleados se muestran en el cuadro 1. Para la mezcla de reacción se usó el kit “iTaQ™ universal SYBR® Green supermix, (BIORAD, EUA) usando el protocolo estándar sugerido por el fabricante. Todas las reacciones fueron por triplicado y los resultados se analizaron con el método ΔΔ-Ct con curva estándar (Livak, *et al.* 2001). Finalmente, los datos fueron sometidos a una transformación logarítmica y se

realizó un análisis multivariado (ANOVA), considerando el uso de dietas y el tiempo como factores ($p < 0.05$).

Cuadro 1. Secuencia de los cebadores utilizados.

Gen	Secuencia (5'-3')	pb	GenBank	Fuente
ProPo	F:TTCCAGCTCTTCTTCATGCT	116-9	EF115296	-----
	R:TCGGGGTACTTGGCGTCCTG		EU373096	
BGPB	F:TCCATTTGAAAGGGCAGAAC	77	EU102286	-----
	R:CATCCCCGAAATTCACCTT			
L8	F:TAGGCAATGTCATCCCCATT	167	DQ316258	Gómez-Anduro <i>et al.</i> 2006
	R:TCCTGAAGGGAGCTTTACACG			

Los resultados mostraron que la incorporación de bioflóculos a la alimentación del camarón tuvo un efecto muy significativo en la expresión de los dos genes considerados (Figura 13).

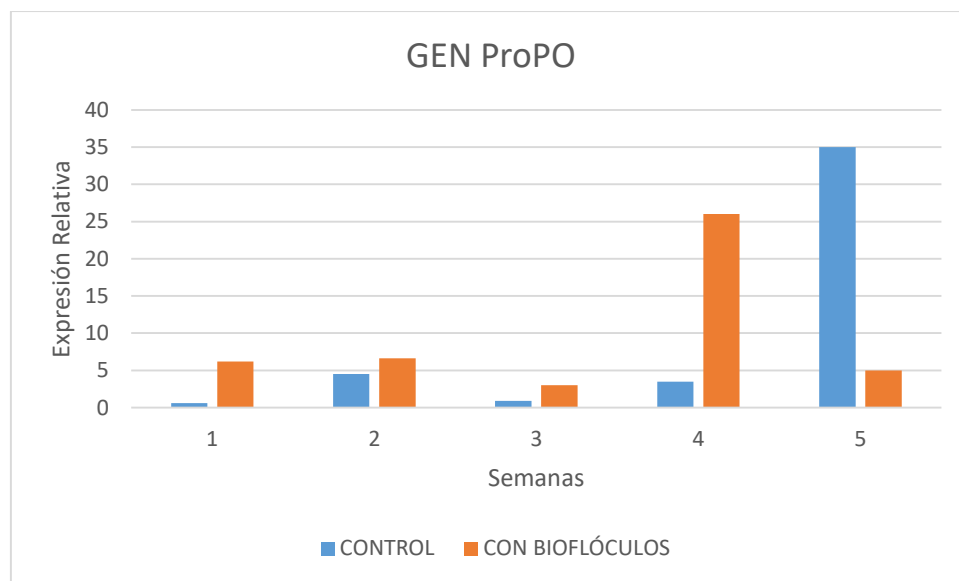


Figura 13. Expresión relativa de los genes BGPB y ProPO en camarones control y alimentados con bioflóculos a la semana uno (1), dos (2), tres (3); 72 horas post-infección con WSSV (4) y 72 horas post-infección con *V. parahaemolyticus* (5)

Esta expresión de genes se vio reflejada en la supervivencia de los camarones sometidos al reto con *Vibrio parahaemolyticus* y virus de la mancha blanca (WSSV) tal como se puede observar en la Figura 14.

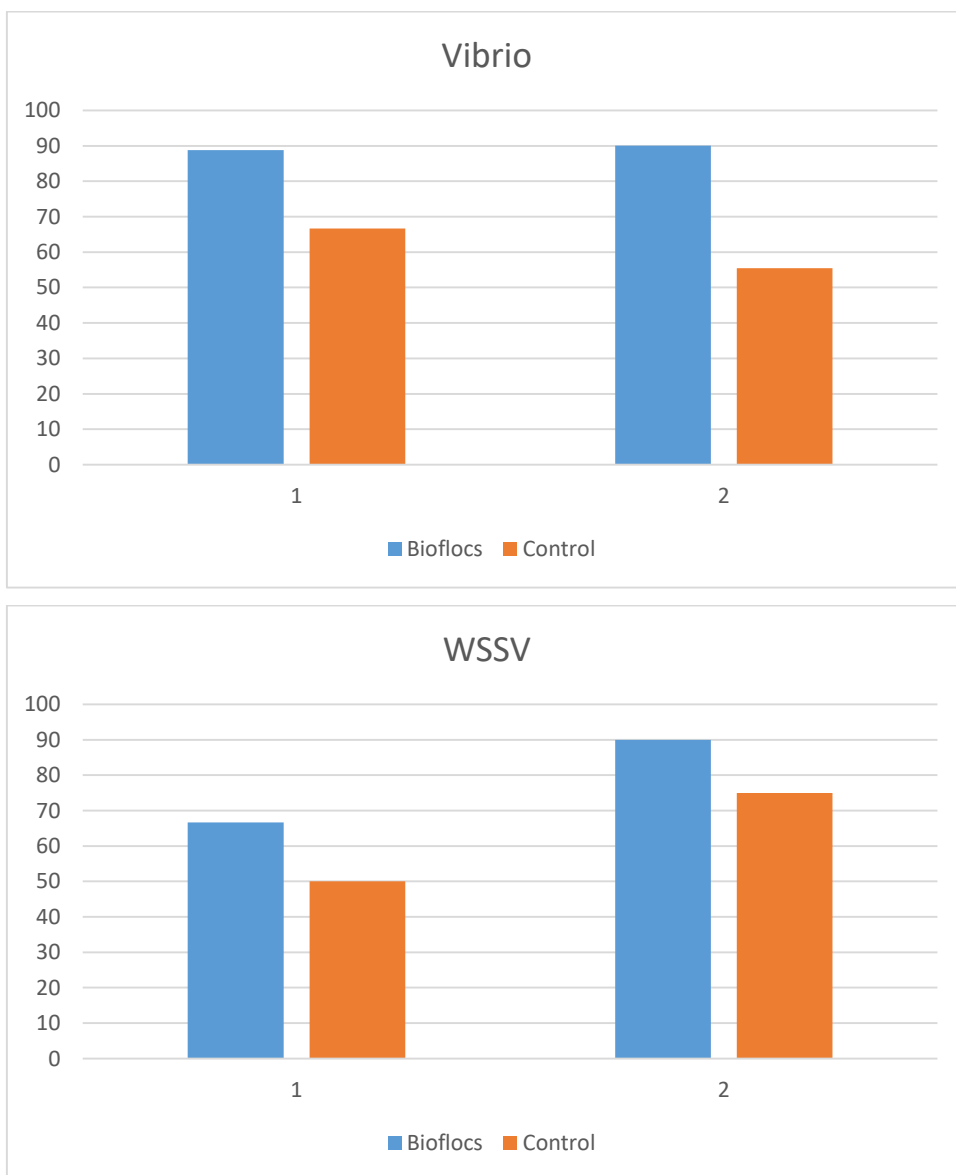


Figura 14. Supervivencia de *L. vannamei* alimentado con y sin bioflóculos, antes y después de los retos con *V. parahaemolyticus* y WSSV

Conclusiones

Con los resultados hasta hoy obtenidos es posible hacer algunas conclusiones importantes:

1. Aunque la superficie de malla sombra fue seleccionada por mostrar el mejor desempeño en cuanto a la estabilidad y composición de las biopelículas, es factible que otros materiales pudieran ser iguales o mejores para tal propósito.
2. La composición química proximal de las biopelículas obtenidas en malla sombra con la microalga *N. incerta* es muy adecuada para la nutrición del camarón.
3. La introducción de biopelículas y bioflóculos al sistema de cultivo, mejora la calidad del agua, sobre todo en términos de concentración de nitrógeno amoniacal.
4. El consumo de microorganismos asociados a biopelículas o bioflóculos, ya sea basados en organismos foto-autotróficos o heterotróficos, mejora la respuesta productiva del camarón.
5. El consumo de estos consorcios microbianos modifica la composición del microbioma intestinal del camarón lo cual puede traer efectos benéficos al hospedero. Esto es necesario investigarlo aún más.
6. Los microorganismos asociados a biopelículas y bioflóculos, consumidos por el camarón modifican la expresión relativa de algunos genes relacionados con el sistema inmune y esto se traduce en mejor resistencia a patógenos.

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La Biomasa Microbiana como Ingrediente en la Nutrición Acuícola

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Resumen

Los productos derivados de la pesca y la acuicultura tendrán un papel primordial en la satisfacción de las necesidades alimentarias de la creciente población humana. La harina de pescado utilizada para la manufactura de alimentos acuícolas representa un recurso limitado que experimenta alta demanda y una serie de debates ambientales. Entre las diversas fuentes alternativas de nutrientes, la biomasa microbiana producida a partir de organismos heterótrofos y autótrofos ha sido considerada como un sustituto prometedor para reemplazar ingredientes derivados de animales y plantas. Diversos estudios han demostrado que algunas especies de levaduras, bacterias y microalgas son candidatos viables para ser cultivados y que además muestran excelentes características nutricionales. Aunque los costos de producción para generar biomasa microbiana aún siguen siendo altos, nuevos métodos se han centrado en la utilización de substratos alternativos para su producción. Las características nutricionales de los microorganismos y las tecnologías emergentes para su producción, permiten pronosticar un mayor uso en la fabricación de alimentos. El presente manuscrito revisa el estado actual del uso de microorganismos como ingredientes en la nutrición acuícola, enfatizando aquellos que muestran un sólido potencial como aditivos funcionales y/o ingredientes para reemplazar la harina de pescado. Se presenta una síntesis de técnicas de evaluación nutricional aplicadas para evaluar el desempeño de la biomasa microbiana, así como resultados recientes sobre los efectos de su incorporación en dietas formuladas. La capacidad fisiológica que presentan diversas especies de organismos acuáticos para utilizar este tipo de insumos alternativos es discutida.

Palabras clave: biomasa microbiana, nutrición acuícola, levaduras, microalgas, bacterias.

Introducción

El rápido crecimiento de la industria acuícola puede ser atribuido en gran parte al mejoramiento y diversificación de las técnicas de producción; sin embargo, los parámetros de producción superiores e intrínsecos de los animales acuáticos también han jugado un papel importante en el desarrollo de esta actividad. Diversas especies de crustáceos y peces convierten el alimento suministrado en proteínas de manera mucho más eficiente que otros animales, tales como las aves de corral, el ganado vacuno y el porcino. Por esta razón se pronostica que diferentes especies de peces, moluscos y crustáceos representarán fuentes predominantes de alimento en el futuro cercano (Béné *et al.* 2015). Conforme la industria acuícola se desarrolla y los métodos de producción se intensifican y diversifican simultáneamente, la demanda de alimentos para dicha industria aumentará aún más. A su vez, ocurrirá un concomitante aumento en la demanda de ingredientes. Pronósticos confiables indican que el aspecto más crítico que puede frenar o incluso detener el actual crecimiento de la industria acuícola es la restricción de los ingredientes requeridos para la manufactura de alimentos (Tacon & Metian 2008, Olsen & Hasan 2012). Entre estos ingredientes, la harina de pescado ha sido el componente preferido al formular alimentos balanceados gracias a sus propiedades nutricionales para los organismos acuáticos. Sin embargo, la producción de harina de pescado trae consigo preocupaciones económicas y ambientales. En consecuencia, esfuerzos para reemplazar, total o parcialmente este insumo en las dietas acuícolas, se desarrollan actualmente y constituyen pasos importantes hacia el uso de fuentes de proteínas sustentables y alternativas. Ingredientes alternativos son cada vez más solicitados en respuesta al crecimiento en la demanda de alimentos acuícolas. Una gran cantidad de estudios nutricionales se han enfocado en probar fuentes de proteínas alternativas poco convencionales y entre las últimas, las proteínas de origen vegetal han sido usadas tradicionalmente para reemplazar la harina de pescado. Sin embargo, las proteínas vegetales pueden no ser totalmente adecuadas para algunas especies acuáticas depredadoras (Kumar *et al.* 2012), además de que pueden existir conflictos respecto a los usos finales de los productos agrícolas. Estas restricciones, junto con los rápidos avances en diferentes áreas de la biotecnología, permiten suponer que la próxima generación de

proteínas útiles para dietas animales estará significativamente representada por proteínas e ingredientes derivados de microorganismos.

El término “proteína unicelular” (single cell protein, SCP, por sus siglas en inglés) fue acuñado en 1966 por C. L. Wilson en el Instituto Tecnológico de Massachusetts (Doelle 1944). SCP se refiere colectivamente a la biomasa procesada o extraída de cultivos de microorganismos que tienen un alto potencial para ser utilizados como ingredientes para alimento humano o alimento animal. Las sobresalientes ventajas de los microorganismos para la producción de SCP, en comparación con fuentes convencionales de nutrientes, han sido ampliamente demostradas (Becker 2007; Øverland *et al.* 2010; Dewapriya & Kim 2014). Los microorganismos crecen rápidamente y tienen un alto rendimiento. Por ejemplo, se ha estimado que se puede obtener una ganancia de 1 kg de proteína en 1 día de crecimiento de un bovino de 500 kg, mientras que 500 kg de levadura producirían varias toneladas de proteína en un día. Microalgas cultivadas en estanques pueden producir más de 20 toneladas de proteína por acre al año (Weyer *et al.* 2010). En comparación, este rendimiento es de 10 a 15 veces más alto que la producción estándar de soya, y 25 a 50 veces más alto que la producción de maíz (Pelczar & Chan 2010). Los microorganismos tienen un alto contenido proteico y son altamente eficientes al convertir una gran variedad de sustratos, lo que conduce a la rápida producción de biomasa. Por otro lado, los microorganismos pueden también clasificarse de acuerdo a las fuentes de carbono y energía que son capaces de utilizar. Dicha diversidad metabólica microbiana es clave y ha permitido la producción de biomasa usando una amplia gama de fuentes de energía disponibles (luz solar o luz artificial) y sustratos de carbono, que van desde monosacáridos de grado analítico hasta residuos generados por las industrias agrícolas y relacionadas a la producción de alimentos, así como también a partir de gas metano. Las técnicas de producción se pueden adaptar a esta diversa variedad metabólica microbiana, y gracias a la ingeniería genética, también es posible lograr lo contrario, es decir, adaptar estos microorganismos a las técnicas de producción actuales y disponibles (Gómez-Pastor *et al.* 2011). Aunque no específicamente producidas para la acuicultura, la generación continua de biomasa microbiana en fermentadores (levaduras) y foto-biorreactores, (microalgas) ha alcanzado una escala industrial. Una serie de metodologías estandarizadas (Fig. 1) permiten predecir un significativo incremento en el número de aplicaciones en la nutrición acuícola.

Una serie de nuevos métodos de producción, independientes de las condiciones estacionales ya han sido patentados y pueden generar biomasa microbiana a partir de substratos alternativos (Glencross *et al.* 2014; Goodall *et al.* 2016). Algunos de estos métodos alternativos han sido capaces de mantener una producción continua y bromatológicamente consistente de biomasa microbiana a partir de microalgas, levaduras y bacterias. La biomasa que se genera puede llegar a representar el producto final de un proceso específicamente establecido, o bien, esta biomasa puede conformar un sub-producto de un proceso primario, como lo es el caso de la producción de biocombustibles o pigmentos a partir de microalgas.

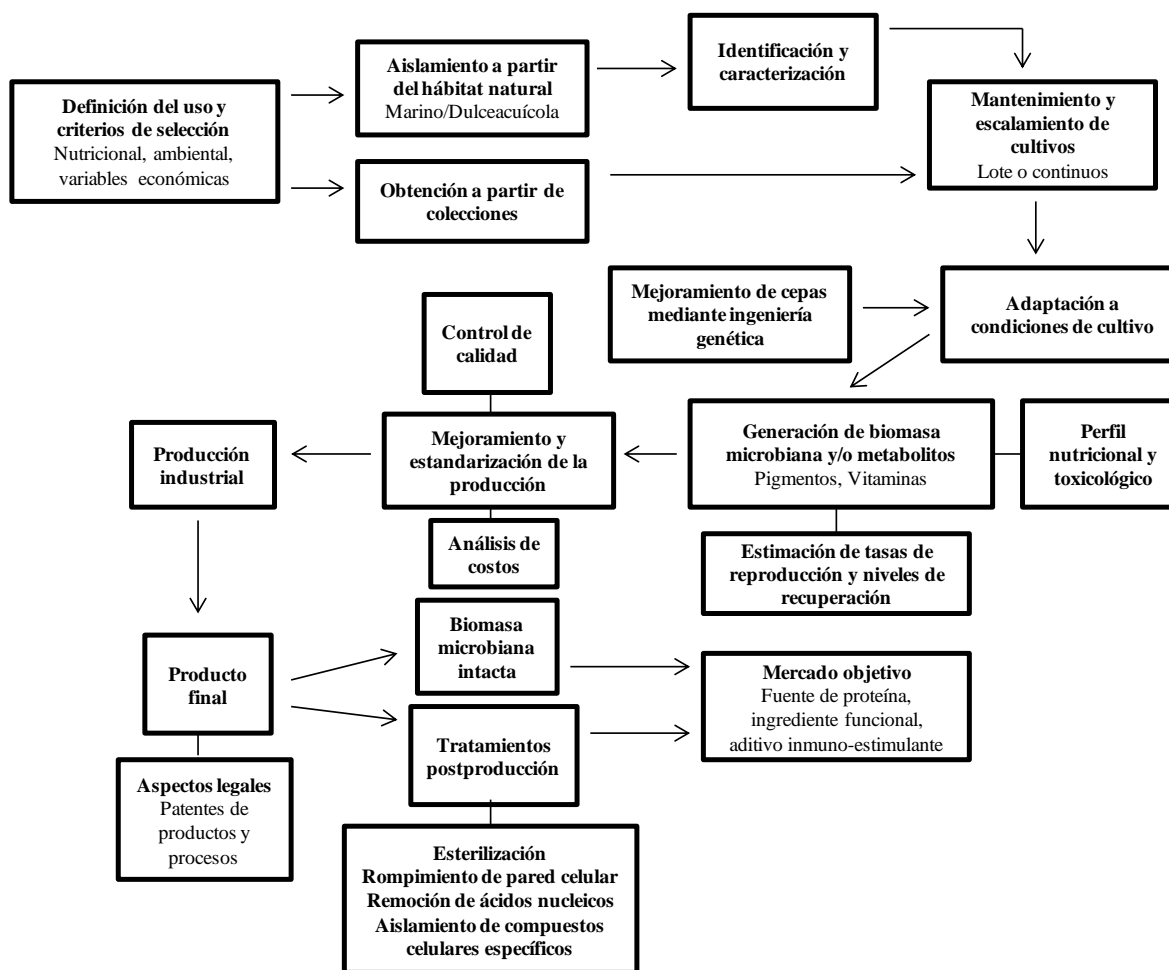


Figura. 1. Principales etapas en la producción de microorganismos que presentan potencial para generar biomasa microbiana o compuestos específicos útiles en la nutrición animal.

La biomasa bacteriana ha sido dirigida a una amplia variedad de propósitos. Por ejemplo, en el caso de la nutrición humana, el hongo unicelular *Fusarium venenatum* ha sido producido durante cuatro décadas para manufacturar un amplio rango de productos comerciales (Quorn®) a partir de una micoproteína. Por otro lado, diversas microalgas tales como *Spirulina* y *Chlorella* tienen actualmente un nicho de mercado establecido como suplementos dietarios. En la alimentación animal, productos microbianos, principalmente derivados de levaduras, son suministrados en dietas formuladas como fuente de vitaminas, pigmentos, proteínas y aminoácidos específicos. También se ha demostrado su rol como agentes mejoradores de la palatabilidad y potenciadores de la respuesta inmune. Recientemente se ha demostrado que la biomasa bacteriana derivada de la fermentación de gas natural (cultivos bacterianos de *Methylococcus capsulatus*) es una fuente prometedora de proteína debido a su composición de aminoácidos, alta digestibilidad y efectos promotores de crecimiento y mejoramiento del estado salud en animales acuáticos y terrestres (Øverland *et al.* 2010; Romarheim *et al.* 2011). El conocimiento generado por estos y otros estudios sobre esta especie de bacteria (Aas *et al.* 2006) ha promovido el desarrollo de una biomasa microbiana comercial registrada como Bioprotein® (Dansk Bioprotein). Eventualmente, la compañía Calysta (Menlo Park, CA, USA) adquirió la tecnología de producción y desarrolló una biomasa bacteriana que ha sido considerada como una fuente sostenible de proteínas de alta calidad (FeedKind®). Existen otras fuentes de biomasa bacteriana que han sido exploradas recientemente en la nutrición animal. Por ejemplo, Zhao *et al.* (2012) reportan que un 25% de reemplazo de proteína de soya por harina de origen bacteriano no afectó el metabolismo del nitrógeno en cerdos. Zhang *et al.* (2013) demostraron que una SCP (obtenida a partir de *Corynebacterium glutamicum*) fue capaz de reemplazar el 50% de la harina de pescado en dietas para cerdos y generó un crecimiento, digestibilidad de nutrientes y morfología intestinal similares a aquellos registrados en animales alimentados con una dieta que contenía sólo harina de pescado como fuente de proteína. De manera similar, empresas recientemente establecidas se han centrado en producir biomasa bacteriana a partir de sustratos alternativos y económicos (algunos sin costo), empleando métodos innovadores de producción de biomasa microbiana tales como el uso de metano y desechos derivados de las actividades agrícolas y de las industrias de los alimentos (Glencross *et al.* 2014, WEF 2015, BFD 2015). Estas

investigaciones y sus procesos patentados subsiguientes, han establecido la base de productos comerciales como Profloc[®] (Nutrinsic) y FeedKind[®] (Calysta), los cuales han sido dirigidos al sector de la nutrición animal como ingredientes para reemplazar la harina de pescado.

La producción intensiva de diferentes especies de microalgas marinas ha sido una actividad intrínseca de varias operaciones acuícolas. Por ejemplo, la alimentación larval y el enriquecimiento del alimento vivo con microorganismos son fuertemente dependientes del abasto de microalgas; sin embargo, en las últimas décadas, varias empresas se han orientado a producir biomasa de microalgas para la producción de biocombustibles. La demanda de fuentes alternativas de energía ha llevado al desarrollo de bio-refinerías, las cuales se definen como instalaciones en las que se integran procesos y equipos con el propósito de convertir biomasa orgánica en biocombustibles, energía y productos químicos específicos (Cherubini 2010; Singh & Gu 2010). Otros sectores industriales han desarrollado tecnologías para generar biomasa de microalgas para la generación de productos funcionales específicos como carotenoides y ácidos grasos poli-insaturados. Por ejemplo, la microalga *Haematococcus pluvialis* se ha producido intensivamente en fotobiorreactores para generar biomasa y extraer astaxantina bajo métodos comercialmente viables (Olaizola 2000, 2003). El subproducto principal de los procesos de extracción de biocombustibles y pigmentos es una biomasa de microalgas de la cual se extrajeron los lípidos. Biomasa secundaria de este tipo ha sido probada con éxito como una fuente adecuada de proteína en dietas para crustáceos y peces (Ju *et al.* 2012; Kissinger *et al.* 2016). En el caso de productos comerciales derivados de levaduras y destinados a la alimentación animal (Phileo[®], NuPro[®]), estudios han demostrado que incluso una baja inclusión de estas fracciones de levadura (mezclas de péptidos, nucleótidos y otros componentes citoplasmáticos) en las dietas, promueven un mejor crecimiento y un aumento en los indicadores inmunológicos en peces (Lunger *et al.* 2006, Berto *et al.* 2015). Dado que la industria de la acuicultura es una actividad relativamente reciente, existe un conocimiento incompleto acerca de los efectos fisiológicos que estos nuevos ingredientes derivados de microbios promueven en los organismos acuáticos.

Fuentes de proteína animal, vegetal y microbiana para la acuicultura

Las harinas derivadas de productos animales como la harina de pescado y las harinas de subproductos avícolas y bovinos mantienen una disponibilidad continua y ofrecen buenos perfiles nutricionales para la mayoría de especies acuáticas cultivables. Las proteínas marinas obtenidas a partir de calamar, camarón y pescado presentan características nutricionales superiores, pero la manufactura de harinas de origen marino ha generado preocupaciones en relación a la conservación ecológica y en relación a aspectos económicos (Phillips 2005). Las harinas de origen vegetal son las fuentes de proteína más utilizadas para sustituir harinas de origen animal en alimentos acuícolas y su inclusión es cada vez mayor debido a su disponibilidad, costo y a la calidad de los perfiles de aminoácidos (de Francesco *et al.* 2004; Kaushik *et al.* 2004; Cruz-Suárez *et al.* 2009). Además de promover altas tasas de supervivencia y crecimiento, ha sido demostrado que el nitrógeno dietario suministrado por ingredientes derivados de plantas, es fisiológicamente incorporado en altas proporciones en el tejido muscular de camarones (Martínez-Rocha *et al.* 2012; Gamboa-Delgado *et al.* 2013). Las proteínas vegetales, por lo tanto, presentan ventajas nutricionales para los organismos acuáticos; sin embargo, predicciones generadas a partir de la reducción de tierra cultivable, y una creciente demanda de productos agrícolas por la creciente población humana, indican un límite o conflicto en la producción agrícola que es exclusivamente orientada a la producción animal. Adicionalmente, algunas especies acuáticas son menos tolerantes que otras a la presencia de altas proporciones de harinas vegetales en sus respectivas dietas (Francis *et al.* 2001). Entre algunas de las características negativas de las harinas de origen vegetal, se incluye la presencia de anti-nutrientes, algunas restricciones en ciertos aminoácidos y generación de enteritis en peces carnívoros. Lo anterior ha limitado ligeramente su uso y se han incrementado los costos debido al procesado adicional para neutralizar los compuestos anti nutricionales o bien para mejorar los perfiles nutricionales. En este contexto, las características nutricionales de varios tipos de microorganismos han generado interés para implementar su uso como ingredientes en dietas balanceadas. La biomasa microbiana generada a partir de diferentes fuentes puede usarse como aditivo (atractante o suplemento) o como ingrediente para sustituir parcialmente otras fuentes de proteína en dietas balanceadas, por ejemplo, en el caso de la

acuicultura, la harina de pescado y la harina de soya. Estudios relativamente recientes han demostrado que la biomasa microbiana intacta, procesada, o los productos específicos extraídos de levaduras, bacterias y microalgas, promueven efectos importantes sobre el crecimiento y generan una modulación inmunológica en larvas y juveniles de organismos acuáticos (Daniels *et al.* 2010; Biswas *et al.* 2012; Macias-Sancho *et al.* 2012; Vidakovic *et al.* 2016). Por otro lado, también se ha reportado que las levaduras y las bacterias fotosintéticas pueden ser exitosamente producidas y usadas para el cultivo de zooplancton y para reemplazar la harina de pescado (Kim & Lee 2000; Olvera *et al.* 2002; Loo *et al.* 2013; Gamboa-Delgado *et al.* 2016). Las tecnologías asociadas a la acuicultura están evidentemente intensificándose y por lo tanto, promoviendo mayores densidades de siembra. Una eficiente respuesta inmune en los animales cultivados representa un aspecto crucial para mantener y mejorar las tasas de crecimiento y la resistencia al estrés en tales sistemas de producción intensiva. En este contexto, el uso de la biomasa microbiana como un agente promotor del crecimiento y salud animal ha generado mayor interés respecto a sus efectos adicionales.

Valor nutricional de los microorganismos y sus componentes celulares

En general, las células completas de microalgas, levaduras y bacterias contienen un alto contenido de proteína en base seca (Tabla 1). El contenido proteico y los perfiles de aminoácidos de muchos microorganismos han sido reportados y algunos de estos son considerados como portadores de un alto valor biológico (Loosli & McDonald 1968, FAO/WHO 1973, Becker 2007). El contenido de lípidos tiende a ser significativamente mayor en la biomasa derivada de microalgas que en la biomasa de bacterias y levaduras. Mientras que las dos últimas son ricas en proteína, pigmentos, co-factores biológicos y vitaminas. Adicionalmente, un aspecto muy importante que ha sido reportado consiste en que el perfil nutricional de bacterias y microalgas puede ser significativamente modificado al usar medios de cultivo específicos y condiciones particulares de crecimiento (Loo *et al.* 2013; Huang & Su 2014). La pared celular de las levaduras está constituida por 25 a 32% del peso celular (Ferreira *et al.* 2010) y algunos tipos de bacterias tienen paredes celulares que resultan más digeribles que aquellas encontradas en las microalgas y levaduras

(Kobayashi & Kurata 1978). A pesar de que las paredes celulares pueden resultar indigeribles para algunos organismos consumidores (Skrede *et al.* 1998; Liu *et al.* 2016), estas también representan una importante fuente de nutrientes debido a que se componen principalmente de polisacáridos (85-88%) y proteína (13%) (Nguyen *et al.* 1998). Compuestos lipídicos tales como lipoglicanos también están presentes en las paredes celulares microbianas, aunque en menores cantidades (Ginsberg *et al.* 2008). Cuando se compara con otros ingredientes disponibles, la biomasa microbiana representa una fuente insuperable de ácidos nucleicos. Por ejemplo, Rumsey *et al.* (1992) reportaron que del 12 al 20% del nitrógeno en la levadura de cerveza *Saccharomyces cerevisiae* (una fuente microbiana ya utilizada en algunas dietas animales), puede estar constituido por nitrógeno derivado de su ácido ribonucleico (ARN). Los efectos de la suplementación de nucleótidos sobre el crecimiento, las respuestas al estrés e inmunológicas, y la palatabilidad de las dietas han sido escasamente estudiadas durante los últimos 30 años (Oliva-Teles 2012; Li & Gatlin 2006 y referencias en citado artículo). Las ventajas y desventajas nutricionales de los nucleótidos son abordadas en las siguientes secciones.

Tabla 1. Características nutricionales generales de microalgas, bacterias, levaduras y harina de pescado. Rangos de composición reportados en base seca.

	Proteína	Lípidos	Cenizas	Ácidos nucleicos
Microalgas	6-62	3-45	8-43	3-8
Levaduras	45-55	1-6	5-10	6-15
Bacterias	50-65	1-3	3-7	8-12
Harina de pescado	59-74	8-14	10-22	0.2-0.6

Tomado de Miller & Litsky 1976; Brown *et al.* 1997; Becker 2007, Villarreal-Cavazos 2010; Adedayo *et al.* 2011; NRC 2011; Bi & He 2013; Huang & Su 2014.

Biomasa microbiana como aditivo y como sustituto de la harina de pescado

Microalgas

Microalgas marinas y dulceacuícolas han sido extensivamente producidas como parte de las operaciones de larvicultivo de peces, crustáceos y moluscos. Métodos específicos y bien establecidos han permitido la producción continua de varias especies de microalgas en salas de eclosión y en salas de larvicultivo. Sin embargo, la aplicación de las microalgas como ingredientes en acuicultura no es aún extensiva en parte debido a que la intención de estas técnicas de producción es generar biomasa microalgal sin tratar, es decir, no existe el objetivo de extraer y aislar las proteína de las microalgas (Becker 2007). En contraste, la producción de SCP a partir de varios microorganismos, en particular bacterias y microalgas ha recibido mayor atención y esfuerzos en cuanto a su procesado. Las razones principales que han conllevado a esto son de naturaleza económica dado que la mayoría de los métodos de producción de microalgas todavía demandan un alto número de recursos. Por otro lado, varias dificultades técnicas (inconsistencia en la cantidad y calidad nutricional, alta demanda de energía eléctrica) asociadas a los procedimientos de producción de biomasa de microalgas todavía esperan solución (Nasseri *et al.* 2011). Estudios sobre la factibilidad de aplicación de la SCP derivada a partir de microalgas en nutrición animal y estudios sobre el cultivo de microalgas exclusivamente como fuente de proteína, son muy escasos (Fábregas *et al.* 1985; Mahasneh 1997). No obstante, y en vista de que las proteínas derivadas de las microalgas presentan alta digestibilidad, altos valores biológicos y son comparables a las proteínas vegetales usadas convencionalmente (Becker 2007; Teimouri *et al.* 2013), es posible predecir que en algún punto se iniciará la inclusión dietaria de biomasa de microalgas en los alimentos balanceados. Muchos de estos productos provendrán de bio-refinerías y de industrias que generan microalgas para aislar pigmentos y compuestos específicos, produciendo de esta forma, sub-productos valiosos para la nutrición animal. Especies de microalgas frecuentemente probadas en bioensayos en los cuales se han incluido como aditivos están representadas por *Haematococcus pluvialis*, *Spirulina*, *Chlorella* y *Schizochytrium*. Las especies que pertenecen al último género, son microalgas bien conocidas y explotadas como una fuente renovable de ácidos grasos altamente insaturados. Métodos de producción alternativos muestran un gran potencial para apoyar

futuros incrementos en la producción de biomasa microalgal. Por ejemplo, varias especies de microalgas (*Schizochytrium*, *Chlorella*, *Pavlova*) comparten la habilidad fisiológica para reproducirse bajo condiciones de oscuridad, pero requieren el soporte de una fuente de carbono (crecimiento heterotrófico), situación que reemplaza el uso tradicional de un suministro de luz natural o artificial (crecimiento autotrófico). Perez-Garcia *et al.* (2011) han reportado que bajo ciertas condiciones de crecimiento heterotrófico, la cantidad de biomasa microalgal producida es consistente y reproducible, alcanzando densidades celulares de 50 a 100 gramos de biomasa seca por litro de medio de cultivo. En comparación, este rango de producción es mucho mayor que el máximo posible alcanzado en cultivos autotróficos (30 gramos por litro) y es similar a la producción de biomasa seca de levadura actualmente utilizada en fermentadores comerciales (130 gramos por litro). Una serie de resultados generados a partir de diversos estudios que han utilizado biomasa derivada de microalgas como ingrediente en dietas acuícolas, indican una mayor cantidad de efectos positivos que negativos sobre el crecimiento, la supervivencia, la pigmentación y la respuesta inmune de los organismos acuáticos (Tabla 2).

Levaduras

Las levaduras representan el primer grupo de microorganismos reconocidos como importantes al ser usados como suplementos en dietas para animales (Nasseri *et al.* 2011). Las levaduras y otros hongos unicelulares contienen altos niveles de proteína y han sido tradicionalmente incluidos en varias formulaciones para animales terrestres. La biomasa de diferentes levaduras ha sido también experimentalmente probada en un amplio rango de animales acuáticos. La principal especie utilizada en la fabricación de levaduras comprimidas es la levadura de panificación *Saccharomyces cerevisiae*, cuya producción depende de cultivos puros y melazas. La primera etapa de producción incluye fermentación en serie en confinamiento. La biomasa obtenida es recuperada del fermentador final por centrifugación y un subsecuente filtrado para concentrar las células. La levadura es mezclada con otros ingredientes y después de una extrusión se comercializa en paquetes compactados o como polvo (EPA 1995). Recientes procesos de post-producción se han enfocado en la remoción de las paredes celulares, para así producir un extracto de levadura

con propiedades nutricionales mejoradas para peces y crustáceos (Vidakovic *et al.* 2016; Zhao *et al.* 2017). La levadura de panificación es comúnmente adicionada a las dietas compuestas para animales como una fuente adicional de aminoácidos, vitaminas y para promover efectos positivos sobre la palatabilidad de las dietas, lo cual puede ser atribuido al alto contenido de nucleótidos en las células individuales de levadura. Previamente se ha reportado que hasta el 20% del nitrógeno total en *Saccharomyces cerevisiae*, puede estar compuesto de bases púricas y pirimidínicas de las nucleoproteínas (Rumsey *et al.* 1992). Aunque una alta digestibilidad de las levaduras ha sido demostrada, las levaduras no pueden ser utilizadas para reemplazar toda la proteína disponible en las dietas para organismos acuáticos. Los niveles de inclusión inicialmente recomendados oscilaban entre 5 y 10% para peces y de 2 a 5 % para camarones (Hertrampf & Piedad-Pascual 2000). Mayores porcentajes de inclusión han sido probados con éxito, pero en niveles cercanos a la sustitución completa de la harina de pescado, se genera menor palatabilidad y menor crecimiento en peces. Rumsey *et al.* (1991) reportaron una disminución en el consumo de alimento en trucha arcoíris alimentada con dietas formuladas con altos niveles de levadura; dietas con contenidos mayores a 50% no fueron aceptadas. Sin embargo, varios estudios recientes han mostrado que los niveles dietarios de levadura pueden incrementarse sustancialmente después de provocar el rompimiento de la pared celular de las levaduras y al garantizar que las dietas se encuentren nutricionalmente bien balanceadas (Tabla 3).

Bacterias y material biofloculado seco

Varias especies de bacterias han generado interés debido a su gran adaptabilidad para ser cultivadas masivamente y también debido a su potencial de uso en acuicultura. Estudios preliminares han reportado acerca de la producción y aplicación exitosa de la bacteria fotosintética *Rhodospseudomonas palustris*, aunque otras especies han sido también investigadas en cuanto a su producción y desempeño nutricional (Kim & Lee 2000; Loo *et al.* 2013). Por otro lado, bacterias metanótrofas producidas con gas natural como fuente de carbono, han sido también investigadas como fuentes alternativas de nutrientes para animales terrestres y acuáticos. Entre estas bacterias, *Methylococcus capsulatus* ha sido probada en diferentes animales monogástricos terrestres y acuáticos (Øverland *et al.* 2010).

Gamboa Delgado, J. et al. 2017. La biomasa microbiana como ingrediente en la nutrición acuícola. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. (Eds), Investigación y Desarrollo en Nutrición Acuicola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 213-263. ISBN 978-607-27-0822-8.

Bioensayos realizados en peces han demostrado que la calidad nutricional de una harina derivada de esta especie de bacteria se desempeña bien como fuente alternativa de proteína para reemplazar la harina de pescado en dietas formuladas para salmón del Atlántico (Aas *et al.* 2006). Varias iniciativas comerciales se han orientado a la producción de biomasa bacteriana a partir de desechos generados por las industrias de los alimentos y agrícolas. Uno de los principales cuellos de botella para estas iniciativas comerciales ha sido la dificultad para estandarizar un producto final nutricionalmente consistente que pueda ser ofrecido como un reemplazo de la harina de pescado o como aditivos dietarios. Sin embargo, ensayos preliminares han demostrado que la inclusión dietaria de esta biomasa microbiana se ha usado exitosamente para promover un mejor crecimiento, supervivencia y respuesta inmune en peces y crustáceos (Tabla 4). Por otro lado, la diversificación de las tecnologías orientadas a la producción de organismos acuáticos ha conllevado al desarrollo de nuevas metodologías encaminadas a promover una mayor producción en menores áreas. Entre estos métodos de producción alternativos, los sistemas basados en el fomento de comunidades microbianas (bioflóculos o biofloc) han sido aplicados para el cultivo de camarón y otros organismos. El principio nutricional de estos sistemas se basa en la formación de material biofloculado, principalmente conformado por agregados bacterianos. Investigaciones recientes sobre este tópico han sido reportadas en varios artículos de revisión e investigación (De Schryver *et al.* 2008; Avnimelech 2009; Emerenciano *et al.* 2012). Los sistemas basados en biofloc pueden ser de naturaleza autotrófica o heterotrófica, dependiendo de la presencia dominante de microalgas, bacterias, o ambas. Una diversa comunidad de microorganismos es frecuentemente fomentada por medio de inóculos y sustratos específicos. El material biofloculado, a su vez, provee sustrato para otros organismos y consiste en una nutritiva fuente adicional de alimento para los organismos en cultivo. Además de tratarse de un alimento de buena calidad y con disponibilidad continua, esta biomasa también ejerce un impacto positivo en la calidad del agua de los entornos de cultivo. Al final de los ciclos de producción en estos sistemas, el material biofloculado remanente puede representar un subproducto útil. De forma similar, las plantas de tratamiento para efluentes de algunas industrias, puede generar importantes cantidades de material biofloculado. Algunos estudios se han dirigido a investigar los efectos de la inclusión de material biofloculado seco en dietas para organismos acuáticos. Tales estudios

proponen aplicaciones nutricionales interesantes para un amplio rango de sub-productos generados por las industrias de los alimentos y bebidas, las cuales generan material microbiano biofloculado durante sus procesos de producción.

Tabla 2. Géneros y especies de microalgas usadas en algunos estudios nutricionales, niveles de inclusión de biomasa y principales efectos reportados en peces y crustáceos.

Organismo de prueba	Microalga y método de producción	Nivel de inclusión dietario	Ventajas	Desventajas	Referencia
Carpín <i>Carassius carassius</i>	<i>Chlorella</i> Estanques abiertos, biomasa liofilizada	0, 75 y 100% de reemplazo de HP	Inclusión de la biomasa de microalga incrementó el crecimiento, la utilización del alimento y la actividad amilasa	Cariopcnosis en hepatocitos	Shi <i>et al.</i> 2017
Dorada <i>Sparus aurata</i>	<i>Scenedesmus almeriensis</i> Foto-reactores tubulares a gran escala	12, 20, 25 y 39 % del peso de la dieta	Todas las dietas produjeron TC similares, utilización de la proteína y actividad enzimática similares. Incremento en capacidad de absorción intestinal en peces que recibieron dietas con microalgas	El análisis de costos actual limita el uso a gran escala de esta microalga en alimentos para peces	Vizcaíno <i>et al.</i> 2014
Trucha arcoiris <i>Oncorhynchus mykiss</i>	<i>Spirulina platensis</i> Producida industrialmente,	2.5, 5.0, 7.5 y 10 % de una dieta de referencia	Sin diferencias significativas en crecimiento y TCA entre tratamientos. Similares contenidos de proteína en musculo	La mayoría de las dietas requirieron suplementación de metionina y lisina Menor contenido de lípidos	Teimouri <i>et al.</i> 2016

	comercialmente disponible		Disminuyó la peroxidación de lípidos en filete al aumentar la cantidad de dietaria de <i>Spirulina</i>	en filete respecto a peces control	
Huayaípe <i>Seriola rivoliana</i>	<i>Haematococcus pluviialis</i> Subproducto derivado de la producción de astaxantina	HP reemplazada por una mezcla de <i>H. pluviialis</i> , aislado de soya y harina de calamar	Sin diferencias significativas en consumo de alimento, crecimiento y TCA en comparación a una dieta con HP, calamar y 5% <i>S. limacinum</i> Mayor retención de lípidos en dietas con 80% de ingredientes reemplazando HP. Menor pérdida de DHA. No se observó enteritis	La mayoría de las dietas requirieron suplementación de aminoácidos Peces alimentados con dieta conteniendo solo HP mostraron mayor retención de nitrógeno	Kissinger <i>et al.</i> 2016
	<i>Schizochytrium limacinum</i> Producida industrialmente	<i>H. pluviialis</i> 2, 6, 11 y 14% de la dieta <i>S. limacinum</i> 5% de la dieta		Los niveles de EPA disminuyeron al disminuir la HP	
Mero <i>Epinephelus lanceolatus</i>	<i>Schizochytrium limacinum</i> Producida industrialmente	20, 40 y 80% de las fuentes de proteína marina contenidas en la dieta	La incorporación de esta microalga que aporta altas cantidades de lípidos permitió reemplazar completamente el aceite de pescado en las dietas con 40 y 80% de reemplazo. Mezcla de ingredientes útil para reemplazar 40%	TC, TCA, consumo de alimento, retención de N y lípidos se redujeron significativamente en peces alimentados con dietas con 80% de biomasa de	García-Ortega <i>et al.</i> 2016

			de las proteínas de origen marino	microalga	
			Crecimiento y utilización de alimento similares entre tratamientos, similares índices hepato-somáticos, aumento del contenido de ácidos grasos con el aumento del nivel de inclusión en la dieta	El análisis de AA en dietas indicó que la metionina fue limitante en la dieta con 80% de biomasa de microalga	
				Enteritis observada a altos niveles de remplazo	
Tilapia <i>Oreochromis niloticus</i>	<i>Spirulina</i> , <i>Chlorella</i> and <i>Schizochytrium</i> Producida industrialmente, comercialmente disponible	30 % de una dieta de referencia conteniendo HP	Los coeficientes de digestibilidad aparente para AA aportados por ambas microalgas fueron similares a los AA de la harina de pescado <i>Spirulina</i> representa una buena proteína alternativa y <i>Schizochytrium</i> puede sustituir el aceite de pescado o los ácidos grasos insaturados	-	Sarker <i>et al.</i> 2016
Lobina europea <i>Dicentrarchus labrax</i>	<i>Isochrysis</i> sp. Foto-bioreactores tipo panel, pared	10 y 20% de la HP disponible en dieta	Similar desempeño en el crecimiento entre tratamientos	Menor digestibilidad de los lípidos y energía en peces recibiendo el mayor nivel de inclusión de microalga	Tibaldi <i>et al.</i> 2015

verde						
Camarón blanco <i>Litopenaeus vannamei</i>	<i>Spirulina</i> sp. and <i>Nannochloropsis oculata</i> Comercialmente disponible	50, 66, 75 y 100% del N dietario suministrado por HP	El reemplazo de 50% de HP mediante cada una de las microalgas, generó TC y supervivencias similares	El reemplazo completo de HP con ambas microalgas causó una reducción significativa en el peso final		Gamboa-Delgado <i>et al.</i> <i>In press</i>
Camarón blanco <i>Litopenaeus vannamei</i>	<i>Schizochytrium</i> y <i>Grammatophora</i> Producida en cultivos en lote	5 y 10 % del N dietario suministrado por HP	Las dietas suplementadas con 5% de <i>Schizochytrium</i> sp. generaron mayor crecimiento que una dieta de referencia (100% HP)	Contenido de cenizas muy alto (50-55%) en ambos tipos de biomasa microalgal		Pacheco-Vega <i>et al.</i> 2017
			La suplementación con <i>Grammatophora</i> sp. generó similares tasas de crecimiento y supervivencia			
Camarón blanco <i>Litopenaeus vannamei</i>	<i>Nanofrustulum</i> <i>Tetraselmis</i> Biomasa sin lípidos generada	25 y 40 % de la proteína suministrada por la HP	Similares TC y tasas de supervivencia	Menor contenido de proteína en animales alimentados con mayores niveles de inclusión de biomasa de microalgas		Kiron <i>et al.</i> 2012

en instalación de biocombustibles					
Camarón tigre <i>Penaeus monodon</i>	<i>Entomoneis</i> <i>Melosira</i> <i>Stauroneis</i> <i>Chlorella</i> <i>Dunaliella</i> <i>Nannochloropsis</i>	5% de la biomasa de cada especie otorgada en peso seco	Tasas de supervivencia mejoradas y significativamente mayor para <i>Melosira sp.</i> Pesos finales, índices de condición y perfiles de ácidos grasos y amino ácidos en músculo similares entre dietas	-	Li <i>et al.</i> 2016
Policultivo de clorofíceas. Aisladas del medio natural y cultivadas					
Camarón blanco <i>Litopenaeus vannamei</i>	Combinación de diferentes especies cultivadas en agua verde	10, 20, 30 y 40 % de la HP	Similares factores de conversión alimenticia en los tratamientos al comparar con el control. Mayor pigmentación en musculo al aumentar el nivel de microalgas en dietas	Las TC de los animales disminuyeron al incrementar los niveles dietarios de microalgas	Basri <i>et al.</i> 2015
Camarón blanco <i>Litopenaeus vannamei</i>	<i>Haematococcus pluviialis</i> Subproducto de la	12.5, 25, 37.5 y 50% de la HP	Reemplazo de 12.5% incrementó la tasa de crecimiento y redujo la TCA. Mayor pigmentación y niveles de astaxantina libre en musculo	Los efectos de altos niveles de inclusión de microalgas sobre la palatabilidad no se han evaluado	Ju <i>et al.</i> 2012

	extracción de astaxantina				
Camarón blanco <i>Litopenaeus vannamei</i>	<i>Arthrospira</i> (<i>Spirulina platensis</i>) Foto-biorreactores expuestos	25, 50, 75 y 100 % de la HP disponible	Sin diferencias significativas en las TC y supervivencia entre animales alimentados hasta con 75% de reemplazo de HP. Una sustitución del 25% incrementó el número de hemocitos granulares circulantes	La sustitución completa de HP conllevó a una reducción en las tasas de crecimiento, aumento en la TCA y deficiencias en AA	Macias-Sancho <i>et al.</i> 2014

HP: Harina de pescado, TC: Tasas de crecimiento, TCA: Tasas de conversión alimenticia, AA: Aminoácidos, N: Nitrógeno

Tabla 3. Diferentes tipos de biomasa de levaduras y sus efectos como ingredientes alternativos en alimentos acuícolas experimentales para peces y crustáceos

Organismo de prueba	Levadura usada y método de producción	Nivel de inclusion dietario	Ventajas	Desventajas	Referencia
Tilapia del Nilo <i>Oreochromis niloticus</i>	Extracto de levadura <i>Saccharomyces cerevisiae</i> Mezcla orgánica certificada como fuente de proteína y aditivo funcional.	1, 2, 4, y 8% de la dieta	Incremento de 33% en el consumo de alimento y 29% en TC en animales alimentados con el nivel mayor de reemplazo. Supervivencia, TCAy retención de proteína no fueron afectadas. Peces desafiados con <i>Aeromonas</i> mostraron similar supervivencia y respuesta inmune que animales control	Disminución lineal de los coeficientes de digestibilidad de la proteína y energía al aumentar la suplementación con levadura	Berto <i>et al.</i> 2015
Trucha alpina <i>Salvelinus alpinus</i>	<i>Saccharomyces cerevisiae</i> Levadura no procesada Extracto de	Cada ingrediente reemplazó el 40% de HP	TC, retención de proteína y AA no fueron afectados en peces recibiendo cada tipo de ingrediente en relación a peces control Similares TC y digestibilidad en peces recibiendo <i>Rhizopus oryzae</i>	Peces alimentados con levadura intacta exhibieron menor digestibilidad para proteína y AA Reducción en la función de barrera intestinal	Vidakovic <i>et al.</i> 2016

	levadura		<i>S. cerevisiae</i> sin procesar aparenta ser una promisorio fuente de proteína para este pez		
	Hongo <i>Rhizopus oryzae</i>				
Cobia <i>Rachycentron canadum</i>	Mezcla certificada orgánica usada como fuente de proteína y aditivo funcional: nucleótidos y péptidos.	Proteína derivada de levadura reemplazando HP a 25, 50, 75 y 100%	Cobias alimentadas con la dieta conteniendo 25% de proteína de levadura mostraron similares TC y TCA que los peces control (100% HP)	La TC se redujo y aumentó la TCA al incrementar los niveles dietarios de proteína derivada de levadura por arriba del 25%	Lunger <i>et al.</i> 2006
Tilapia <i>Oreochromis mossambicus</i>	Levadura <i>Torula Candida utilis</i> Comercialmente disponible	25, 30, 35, 40 y 45% de la proteína dietaria	Mejor TCA, TEP y utilización del N al usar dietas con 25% de levadura torula. Una inclusión de 30% generó mayores TC. Digestibilidad de la proteína mayor a 80%. Posible reemplazar hasta 65% de la proteína animal con una mezcla de proteínas vegetales, incluyendo 30% de torula, sin efectos adversos en cultivo y beneficio económico	-	Olvera-Novoa <i>et al.</i> 2002
Tilapia del Nilo	<i>Kluyveromyces</i>	15 % del peso	Similar contenido de lípidos en	Mayor peso final en dietas	Ribeiro <i>et al.</i> 2014

<i>Oreochromis niloticus</i>	<i>marxianus</i>	de la dieta	hígado y músculo	control	
	Fermentación discontinua		Mayor concentración de proteína en el filete. Similar contenido de AGAI en filete		
Lobina híbrida <i>Morone chrysops</i> x <i>M. saxatilis</i>	Levadura de panificación <i>Saccharomyces cerevisiae</i>	1, 2 y 4 % de la dieta	Ganancia de peso y TCA se incrementaron en peces alimentados con dietas suplementadas Exposición a <i>Streptococcus</i> no causó mortalidad. Menores signos de enfermedad en peces recibiendo dietas con 2 y 4% de levadura	-	Li & Gatlin 2003
Panga <i>Pangasianodon hypophthalmus</i> x <i>Pangasius bocourti</i>	<i>Saccharomyces cerevisiae</i> Compañía cervecera	30, 45, 60 y 75 % de la HP disponible	El peso final y la TCA indicaron que la levadura puede usarse para reemplazar hasta 45% de la HP Similares eficiencias de alimentación, perfil hematológico y calidad de carne entre los tratamientos Incremento de la actividad lisozima	-	Pongpet <i>et al.</i> 2015

Camarón blanco <i>Litopenaeus vannamei</i>	Levadura <i>Torula Candida utilis</i> Producida industrialmente	7.5, 15, 30 y 60 % del nitrógeno dietario aportado por HP	Similares tasas de supervivencia entre tratamientos. Mayor TC en animales alimentados con dietas conteniendo levadura y HP que en animales alimentados con dietas con HP Asimilación del N y materia seca total a partir de la levadura torula se incrementó en relación al aumento de proporciones dietarias Levadura apta para sustituir hasta 60% de la HP	Costos de producción de este ingrediente aún son relativamente altos. Disminución en la incorporación de N en tejido muscular a un nivel de reemplazo del 60%	Gamboa-Delgado <i>et al.</i> 2016
Camarón blanco <i>Litopenaeus vannamei</i>	Extracto de levadura aplicado para reemplazar HP Producto comercial	15, 30, 45, 60 y 100% de la HP	Similar composición del musculo entre tratamientos. Todos los niveles de reemplazo causaron mayor digestibilidad de la proteína que la dieta basal (25% HP) Hasta 45% de la HP puede ser reemplazada por extracto de levadura	-	Zhao <i>et al.</i> 2017
Langosta	Levadura y granos	Reemplazos	No se detectaron diferencias	Se necesita mayor	Muzinic <i>et al.</i> 2004

australiana	de destilería	parciales y	significativas en peso final ni	información acerca de los
<i>Cherax</i>		totales de HP y	supervivencia	requerimientos de AA en
<i>quadricarinatus</i>		harina de		<i>Cherax</i>
		camarón	La HP y la harina de camarón pueden	
			ser totalmente reemplazadas con	
			harina de soya y granos de destilería	
			con levadura	

HP: Harina de pescado, TC: Tasas de crecimiento, TCA: Tasa de conversión alimenticia, TEP: Tasas de eficiencia proteica, AGAI: Ácidos grasos altamente insaturados, N: Nitrógeno.

Tabla 4. Diferentes fuentes de biomasa bacteriana y mezclas complejas microbianas de material seco biofloculado y sus efectos nutricionales al ser incluidos como ingredientes en dietas experimentales para peces y crustáceos.

Organismo de prueba	Material microbiano utilizado y método de producción	Nivel de inclusion dietario	Ventajas	Desventajas	Referencia
Salmón del Atlántico <i>Salmo salar</i>	<i>Methylococcus capsulatus</i> Producida a base de metano como fuente de carbono	30% de una dieta con HP y harina de soya	El consumo de alimento acumulado no fue diferente entre tratamientos La biomasa microbiana puede ser usada para prevenir la enteritis inducida por la harina de soya	La digestibilidad de la proteína cruda y lípidos se redujo con la inclusión de biomasa microbiana	Romarheim <i>et al.</i> 2011
Salmón del Atlántico <i>Salmo salar</i>	<i>Methylococcus capsulatus</i> Producida a base de metano como fuente de carbono	25 y 50% de los AA disponibles	La digestibilidad de la arginina fue mejorada al incrementar los niveles de biomasa microbiana	La digestibilidad del nitrógeno disminuyó al incremental el nivel dietario de biomasa bacteriana	Skrede <i>et al.</i> 1998
Salmón del Atlántico	<i>Methylococcus capsulatus</i>	Harina de proteína	Las TCA fueron significativamente mayores en animales alimentados con	Las TCA fueron significativamente	Aas <i>et al.</i> 2006

<i>Salmo salar</i>	<i>Alcaligenes acidovorans</i> <i>Bacillus brevis</i> <i>B. firmus</i>	bacteriana incluida a 4.5, 9, 18 y 36% de la dieta	dietas conteniendo 18 y 36% de reemplazo El incremento en los niveles dietarios de proteína de biomasa microbiana redujo el N branquial y renal y la energía invertida en mantenimiento Fuente de proteína alternativa viable para reemplazar la HP en alimento para salmón	mayores en los individuos del tratamiento 36% La digestibilidad del N, AA, lípidos, energía y cobre se redujeron significativamente al incrementar los niveles de proteína derivada de biomasa bacteriana	
Pámpano de Florida <i>Trachinotus carolinus</i>	Biomasa bacteriana seca fermentada	0, 4, 9 y 13% de la HP en dieta	No se observaron diferencias significativas en el peso final de los peces Hasta 13% de biomasa bacteriana seca fermentada pudo ser incluida en dietas para pámpano sin afectar negativamente el crecimiento	-	Rhodes <i>et al.</i> 2015
Trucha arcoíris <i>Oncorhynchus</i>	Biomasa bacteriana Comercialmente	Biomasa bacteriana reemplazó 25,	La dieta con 25 % de biomasa bacteriana no influenció las TC, el consumo de alimento, ni la eficiencia de	Reducción en las TC en al usar los mayores niveles de biomasa bacteriana.	Perera <i>et al.</i> 1995

<i>mykiss</i>	disponible	63 y 100% de la HP	absorción al comparar con una dieta con 100% HP	Disminución en la absorción de nitrógeno. Aumento en la excreción de urea	
Camarón tigre <i>Penaeus monodon</i>	Biomasa microbiana/Bioactivo microbiano Novacq™ Biomasa bacteriana producida a partir de sustratos de bajo costo	5 y 10% del peso de la dieta	La inclusión dietaria de biomasa microbiana compensó el reemplazo de HP y aceite de pescado. Mayores TC reportadas en camarones alimentados con dietas conteniendo el bioactivo microbiano	-	Glencross <i>et al.</i> 2014
Camarón tigre <i>Penaeus monodon</i>	Biomasa microbiana/Bioactivo microbiano Novacq™	10% de una dieta suministrada en tres diferentes raciones	La adición de biomasa microbiana a la dieta generó mejoras significativas en el crecimiento (en general, 26% de incremento) en todos los tratamientos	-	Arnold <i>et al.</i> 2016
Camarón tigre <i>Penaeus monodon</i>	Producto bioactivo derivado de biomasa microbiana Novacq™	5.5 % del régimen dietario para maduración	-	La inclusión de biomasa microbiana en las dietas para maduración no mejoró el desempeño	Goodall <i>et al.</i> 2016

reproductivo

Camarón blanco <i>Litopenaeus vannamei</i>	Material biofloculado seco obtenido como subproducto de granjas de camarón	7.5 a 30% de la HP disponible	La supervivencia, ganancia de peso y TCA fueron significativamente mejoradas en mediante el tratamiento con 30% de harina de biofloc	-	Dantas <i>et al.</i> 2016
			Ingrediente referido como una alternativa coso-efectiva derivada de un subproducto		
Camarón blanco <i>Litopenaeus vannamei</i>	Material biofloculado seco obtenido de un sistema experimental para crianza de tilapia	25, 50, 75 y 100% del nitrógeno dietario suministrado por la HP	La dieta que suministró 25% de nitrógeno a partir del biofloc, fue la única que generó crecimiento comparable a la dieta con 100% HP	Las TC disminuyeron al suministrar dietas hasta con 50 y 75% de biofloc. La incorporación del nitrógeno dietario aportado por el biofloc seco al músculo fue menor que el aportado por HP.	Gamboa-Delgado <i>et al.</i> 2017

HP: Harina de pescado, TC: Tasas de crecimiento, TCA: Tasas de conversión alimenticia, AA: Aminoácidos. N: Nitrógeno

Metodologías para evaluar el desempeño nutricional de la biomasa microbiana

Atractabilidad y palatabilidad

Una buena atractabilidad y palatabilidad dietaria son dos características organolépticas que conducen al inicio y continuación de la respuesta alimenticia en la mayoría de los animales acuáticos. La aguda quimo-recepción de peces y crustáceos permite una rápida detección de sustancias de bajo peso molecular, tales como aminoácidos y ácidos nucleicos (Grasso & Basil 2002; Hara 2005; Rønnestad *et al.* 2013). La mayoría de las sustancias que estimulan a las células sensoriales olfativas y gustativas de peces, se caracterizan por tener bajo peso molecular (<1000 Da) y por tener características nitrogenadas y anfóteras (Hara 1993). Todas estas características químicas aplican a los aminoácidos, betaínas y nucleótidos. El alto contenido de nucleótidos en los microorganismos los convierte en agentes eficientes para mejorar la palatabilidad de una dieta. Los extractos de levadura son frecuentemente utilizados en la industria de los alimentos como mejoradores del sabor, en particular debido al alto contenido de ácido glutámico y nucleótidos (*e.g.* guanosin-monofosfato) conocidos por sus propiedades estimulantes ejercidas sobre peces (Ferreira *et al.* 2010; Berto *et al.* 2015; Li & Gatlin 2006); sin embargo, altas concentraciones dietarias de ácidos nucleicos pueden afectar negativamente la palatabilidad de las dietas. Lunger *et al.* (2006) reportaron que juveniles de cobia alimentados con dietas en las cuales la harina de pescado se reemplazó por completo por una proteína de levadura, presentaron bajas tasas de ingestión y bajas tasas de crecimiento.

Digestibilidad

La estimación de los coeficientes de digestibilidad aparente provee un indicador acerca de la disponibilidad de los nutrientes contenidos en un ingrediente específico y por lo tanto puede utilizarse como parte de los criterios de selección de ingredientes alternativos y tradicionales. La información generada a partir de estos coeficientes facilita la selección de ingredientes para mejorar el valor nutricional de los alimentos, así como para disminuir los costos de producción. La digestibilidad de un ingrediente o insumo depende principalmente

de su composición química y de la fisiología digestiva de la especie a la cual se alimenta (Brunson *et al.* 1997). Varios estudios han sido desarrollados para explorar la digestibilidad de diferentes fuentes de biomasa microbiana seca en varias especies de animales acuáticos. En general, estos estudios han demostrado que la biomasa microbiana tiende a mostrar altos coeficientes aparentes de digestibilidad para la materia seca y para la proteína. Por ejemplo, en bioensayos realizados con camarón blanco, se ha demostrado que algunos tipos de levaduras generan coeficientes de digestibilidad aparentes que resultan similares a aquellos obtenidos para la harina de pescado (82 a 86%, para la proteína cruda) (Cruz-Suárez *et al.* 2009; Villarreal-Cavazos 2011). En peces, Oliva-Teles & Goncalves (2001) reportaron que juveniles de lobina marina (*Dicentrarchus labrax*) alimentados con dietas formuladas con levadura *Saccharomyces cerevisiae*, mostraron coeficientes de digestibilidad altos para la materia seca (72 a 86%), proteína (87 a 93 %) y energía (82 a 95%); sin embargo, los coeficientes para materia seca y energía disminuyeron significativamente al aumentar los niveles dietarios de levadura. La fragmentación de la pared celular microbiana incrementa significativamente el valor nutricional de las levaduras en peces y crustáceos. Zhao *et al.* (2017) demostraron recientemente que al reemplazar toda la harina de pescado por un extracto de levadura en dietas para camarón, fue todavía posible mantener altos coeficientes de digestibilidad para la materia seca (81%) y la proteína cruda (93%), tal efecto puede ser atribuido a la remoción de las paredes celulares. Rumsey *et al.* (1991) previamente reportaron un efecto similar en truchas, en donde la digestibilidad del nitrógeno se incrementó al remover todo el material derivado de la pared celular y al aplicar una subsecuente separación del nitrógeno en fracciones de aminoácidos y ácidos nucleicos. En el caso de la biomasa derivada de bacterias y microalgas, Skrede *et al.* (1998) reemplazaron 25 y 50% de los aminoácidos dietarios de un alimento, con proteína derivada de bacterias (*Methylococcus capsulatus*, *Alcaligenes acidovorans*, *Bacillus brevis* y *B. firmus*). Estas bacterias fueron cultivadas usando gas natural como fuente de carbono, para posteriormente evaluar su digestibilidad en salmón del Atlántico. La digestibilidad de los aminoácidos estuvo en un rango de 52 a 92%, y ésta disminuyó consistentemente al aumentar los niveles dietarios de proteína bacteriana, excepto para el aminoácido arginina. En un estudio reciente, Sarker *et al.* (2016) desarrollaron un experimento con tilapia del Nilo y reportaron

una alta digestibilidad de los ácidos grasos (57-95%) y los aminoácidos (86-94%), respectivamente suministrados por biomasa de microalgas *Schizochytrium* y *Spirulina*.

Asimilación y contribución al crecimiento

El origen de los nutrientes suministrados por diferentes ingredientes y su incorporación final en el tejido de los animales en crecimiento pueden ser evaluados mediante varias metodologías analíticas. Aunque algunos métodos aplicados en crustáceos se han basado en la estimación de las eficiencias de asimilación de la materia orgánica (Condrey *et al.* 1972), las técnicas más frecuentemente aplicadas para estimar la asimilación de nutrientes están representadas por metodologías que utilizan mediciones de radio-isótopos o isótopos estables. Tales técnicas isotópicas surgieron en las ciencias geológicas y son ahora utilizadas ampliamente en estudios de ecología y nutrición. En nutrición acuícola, las metodologías isotópicas han permitido elucidar la utilización fisiológica de nutrientes derivados de ingredientes alternativos a la harina de pescado y derivados de fuentes alternativas de proteína para organismos acuáticos (Conceição *et al.* 2007; Le Vay & Gamboa-Delgado 2011). La aplicación de isótopos estables se basa en la determinación de las abundancias naturales de isótopos de carbono y nitrógeno (por ejemplo, ^{12}C y ^{13}C para carbono, ^{14}N y ^{15}N para nitrógeno), entre varios otros elementos. Tales abundancias naturales tienden a ser consistentes en diversos tipos de alimento o ingredientes y estas firmas isotópicas específicas son transferidas a los tejidos de los organismos consumidores. Estas características permiten diseñar estudios orientados a la determinación del origen y destino de nutrientes. Al integrar los valores isotópicos de los animales consumidores y sus dietas en modelos de mezclado isotópico (modelos de balance de masa), es posible obtener un indicador de las proporciones relativas de nutrientes aportadas al crecimiento de un organismo (Phillips & Gregg 2001; Phillips 2012). En una serie de estudios realizados en nuestro laboratorio, se han aplicado análisis isotópicos de nitrógeno ($\delta^{15}\text{N}$), con el objetivo de evaluar en camarón blanco del Pacífico las contribuciones proporcionales de nitrógeno dietario aportado por diversos tipos de proteína microbiana, la cual ha sido suministrada en forma de biomasa de microalgas, levadura y harina de bioflóculos (Tabla 5). En estos bioensayos, los animales son alimentados con dietas formuladas con diferentes

proporciones de una harina de pescado de referencia y diferentes proporciones de ingredientes derivados de biomasa microbiana. Los ingredientes son seleccionados en función de sus propiedades nutricionales, pero también tomando en cuenta sus valores isotópicos naturales de nitrógeno, los cuales deben de ser diferentes a los de la harina de pescado para facilitar subsecuentes estimaciones.

Tabla 5. Eficiencias de asimilación relativas del nitrógeno dietario aportado por diferentes tipos de biomasa microbiana (BM) y aportado por harina de pescado (HP). Valores porcentuales estimados a partir de análisis isotópicos de nitrógeno ($\delta^{15}\text{N}$).

Microorganismo / Biomasa microbiana	Proporción en dieta (%)		Contribución al crecimiento tisular (%)		Referencia
	HP	BM	HP	BM	
Levadura <i>Torula</i>	85	15 ^a	86	14 ^a	
<i>Candida utilis</i>	70	30 ^a	73	27 ^a	Gamboa-Delgado <i>et al.</i> 2016
	40	60 ^a	49	51 ^b	
Microalgas					
<i>Spirulina</i> +	33	33 ^a	36	50 ^b	Gamboa-Delgado <i>et al.</i>
<i>Nannochloropsis</i> <i>oculata</i>		33 ^a		14 ^b	<i>Submitted</i>
Microalgas					
<i>Grammatophora</i>	90	10 ^a	93	7 ^a	Pacheco-Vega <i>et al.</i> <i>In press</i>
<i>Schizochytrium</i>	90	10 ^a	92	8 ^a	
	75	25 ^a	94	6 ^b	

Harina de biofloc					
derivada de un cultivo de tilapia	50	50 ^a	59	41 ^a	Gamboa-Delgado <i>et al.</i> 2017
	25	75 ^a	36	64 ^b	

* Superíndices indican diferencias significativas (pruebas de bondad de ajuste Chi-cuadrada) entre las contribuciones esperadas (dietas) y observadas (tejido muscular) de nitrógeno dietario incorporado desde los diferentes ingredientes de origen microbiano.

Eventualmente, análisis isotópicos de nitrógeno ($\delta^{15}\text{N}$) son aplicados a ingredientes y tejido muscular para estimar las contribuciones proporcionales de nitrógeno dietario suministrado por los ingredientes al crecimiento del camarón. En general, los resultados generados a partir de estos bioensayos indican que las proteínas de origen microbiano son asimiladas rápidamente y además son incorporadas en el tejido muscular en proporciones que son frecuentemente similares a las proporciones de biomasa microbiana incluidas en las dietas experimentales (Tabla 5). La biomasa producida a partir de las microalgas *Spirulina* y *Nannochloropsis* contiene altos niveles de proteína cruda (59 y 43%); sin embargo, solamente el nitrógeno dietario aportado por *Spirulina* contribuyó al crecimiento en una magnitud mayor que la harina de pescado. Lo anterior se debe posiblemente a que el perfil de aminoácidos de la *Spirulina* es más apropiado para el camarón que el perfil de la biomasa de *Nannochloropsis*. Resultados obtenidos de estos y otros estudios han indicado que al sustituir la harina de pescado en altos niveles ó incluso completamente por algún tipo de biomasa microbiana, se genera una disminución en las proporciones de nitrógeno microbiano contribuyente al crecimiento, así como una disminución en las tasas de crecimiento de los animales consumidores. Dietas formuladas con diferentes niveles de levadura *Torula* (7.5, 15, 30, 60%) sustituyendo a la harina de pescado, generaron crecimiento similar o mayor al promovido por una dieta control conteniendo solamente harina de pescado como fuente de proteína. Las proporciones de aporte de nitrógeno dietario al crecimiento muscular fueron similares a las proporciones establecidas en las dietas, excepto para la sustitución de 60% de harina de pescado, en donde la mayor contribución provino de este ingrediente y no de la levadura. La sustitución total de

Gamboa Delgado, J. et al. 2017. La biomasa microbiana como ingrediente en la nutrición acuícola. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. (Eds), Investigación y Desarrollo en Nutrición Acuícola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 213-263. ISBN 978-607-27-0822-8.

harina de pescado por *Torula*, generó una ganancia de peso significativamente menor que el resto de las dietas. En el caso de la harina obtenida a partir de bioflóculos bacterianos, ésta presentó un nivel de proteína cruda de 24%, el cual es relativamente bajo comparado con la biomasa de levadura *Torula*, pero mayor al estimado en algunas microalgas (*Schizochytrium*). El material biofloculado proveniente de diferentes fuentes (granjas acuícolas, plantas de tratamiento) puede ser extremadamente variable en cuanto a sus propiedades bromatológicas.

Inmuno-estimulación

El efecto inmuno-estimulante que muchos aditivos dietarios confieren a los animales consumidores encaja bien en la definición de alimentos funcionales, los cuales se definen como alimentos que generan beneficios fisiológicos adicionales al cubrimiento de los requerimientos nutricionales básicos (Newaj-Fyzul & Austin 2015). Por ejemplo, un alimento funcional puede mejorar el estado de salud y resistencia y por lo tanto reducir la incidencia de una enfermedad. Es ampliamente conocido el hecho de que algunos alimentos presentan propiedades profilácticas y/o inmuno-estimulantes. Como se mencionó antes, la intensificación de las prácticas acuícolas implica incrementos significativos en las densidades de siembra de los organismos. Tales ambientes estresantes requieren soluciones prácticas para reducir los riesgos de enfermedades infecciosas o aquellas asociadas a la producción de hormonas de respuesta al estrés. Adicionalmente, debido a que existe una creciente preocupación relacionada al excesivo uso de antibióticos, se han impuesto prohibiciones al uso de estos en la mayoría de los países europeos en donde se desarrollan actividades acuícolas. En vista de esto, se ha fomentado la investigación sobre los efectos inmuno-nutricionales de varios ingredientes sobre los organismos acuáticos y reviste una gran importancia actual (Ringo *et al.* 2012). Los efectos inmuno-estimulantes (innato y adaptativo) promovidos por nucleótidos derivados de microorganismos han sido ampliamente demostrados en animales acuáticos (Li & Gatlin 2006; Daniels *et al.* 2010; Biswas *et al.* 2012). Devresse (2000) y Li *et al.* (2007) consideran que los nucleótidos son nutrientes clave para el sistema inmune de los camarones. Por lo tanto, la suplementación de estos, o de sustratos que contienen alta cantidad de nucleótidos, tales como las levaduras

y sus derivados, pueden mejorar la resistencia a las enfermedades y mejorar las tasas de crecimiento. Li & Gatlin (2006) han realizado una síntesis acerca de los avances de investigación sobre los nucleótidos en la nutrición de peces. Los autores declaran que aunque diversos productos derivados de levaduras se han utilizado en formulaciones nutricionales para camarones, el papel de los nucleótidos derivados de levaduras (y otros microorganismos) permanece ampliamente sin descifrar.

También existe evidencia que indica que algunos ácidos grasos (DHA, EPA, ARA) derivados de microalgas pueden ejercer efectos inmuno-estimulantes sobre los organismos acuáticos (Carton-Kawagoshi & Caipang 2015). Similarmente, se ha reportado que diversos componentes de las células microbianas presentan propiedades inmuno-estimulantes. Entre estos se encuentran los lipopolisacáridos, β -glucanos, dipéptidos de muramil y preparaciones bacterianas tratadas con calor (Sakai, 1999). Se requiere investigación adicional sobre el papel nutricional de los nucleótidos y también sobre la gran cantidad de componentes derivados de las paredes y citoplasmas microbianos. El desarrollo de inmuno-estimulantes mejorados dependerá de un mejor conocimiento de los mecanismos de acción de estos componentes microbianos sobre las respuestas fisiológicas promovidas en los animales acuáticos.

Desempeño reproductivo

Existe una información muy escasa acerca de los efectos de la adición dietaria de biomasa microbiana sobre el desempeño reproductivo de peces y crustáceos. Goodall *et al.* (2016) reportaron recientemente que la inclusión de biomasa microbiana en dietas para reproductores de camarón tigre (*P. monodon*) no mejoró el desempeño de los reproductores domesticados en términos de madurez gonadal y producción de huevecillos y nauplios. Es bien conocido que los reproductores de peces y crustáceos requieren dietas frescas de alta calidad nutricional, con altos contenidos de proteína y lípidos, en particular ácidos grasos poli-insaturados. Es por lo tanto razonable asumir que varios tipos de microorganismos pueden ser empleados para suministrar estos componentes; sin embargo, esto posiblemente requerirá una combinación de especies microbianas. Por ejemplo, las microalgas

pertenecientes a los géneros *Schizochytrium* y *Haematococcus* presentan un alto contenido de lípidos, mientras que las microalgas *Spirulina* y *Scenedesmus* pueden contener un alto contenido de proteína en base seca (Duong *et al.* 2015; Kissinger *et al.* 2016).

Las levaduras también pueden representar una fuente de nutrientes específicos para organismos reproductores. Koch *et al.* (2011) reportaron que al adicionar 2% de levadura de cerveza a dietas para reproductores de tilapia se obtuvo una mejora significativa en la supervivencia de los alevines en relación a los efectos promovidos por una dieta de referencia conteniendo solamente proteínas de origen vegetal. En el cultivo de moluscos y equinodermos, el uso de especies específicas de microalgas (algunas de estas concentradas como pasta o polvo) es actualmente una práctica común. Esto se debe a que mediante estas, se promueve el desarrollo gonadal y la salud de los reproductores. Chen *et al.* (2012) usaron una mezcla de pescado macerado, yema de huevo, levadura y *Spirulina* para promover exitosamente la maduración gonadal del pepino marino *Apostichopus japonicas*.

Efectos prebióticos y probióticos

La biomasa microbiana derivada de diferentes microorganismos ha sido también extensivamente probada respecto a sus efectos prebióticos y probióticos. Los prebióticos pueden definirse como elementos de la dieta que no son digeribles pero en cambio estimulan el crecimiento o la actividad de uno o más tipos de bacterias en el tracto digestivo. Los probióticos, en cambio, son microorganismos vivos que al ser agregados como suplemento en la dieta, favorecen el desarrollo de flora microbiana. Los entornos de cultivo y el alimento formulado pueden usarse como vehículos para proveer prebióticos y probióticos; en particular, las esporas derivadas de bacterias benéficas son muy viables para ser administradas como suplementos en alimentos formulados. Ha sido reportado que los organismos cultivados en sistemas intensivos muestran un estado de salud y un nivel de supervivencia significativamente mejorado cuando se manipula la microflora del tracto (e incluso la flora microbiana ambiental) con organismos probióticos o mediante el uso de prebióticos (Olafsen 2001; Rengpipat *et al.* 2000). Aunque falta mucha investigación sobre el modo de acción de los probióticos, se cree que sus efectos se deben a interacciones

inhibitorias, competencia por sitios de adhesión y estimulación inmunológica, entre otros. El uso de probióticos ha tomado auge en acuicultura debido a sus efectos sobre la prevención de enfermedades y por la evidente mejora en el estado nutricional de los animales en cultivo. Similarmente, el interés por su uso aumentó como consecuencia de la presión ejercida por desarrollar un tipo de acuicultura libre de terapéuticos y más amigable ambientalmente. Aunque ha sido demostrado que los probióticos viables vivos son mejores que los no viables inactivados por calor (Panigrahi *et al.* 2005), es muy posible que los ingredientes de origen microbiano inculdos en alimentos acuícolas también ejerzan un efecto atenuado pero positivo como probióticos y prebióticos.

Aspectos económicos de la producción de biomasa microbiana

El incremento en la demanda de productos acuícolas y pesqueras ha ejercido una fuerte presión sobre los recursos marinos (McClanahan *et al.* 2015). Esto se ha visto reflejado en incrementos constantes de precio. Por ejemplo, el precio de la harina de pescado se ha incrementado de \$ 600 USD por tonelada en 1995 a \$ 1700 USD por tonelada en 2015 (IM 2016). Claramente se requieren fuentes alternativas de proteína, y esta necesidad ha conllevado a la investigación intensiva. Existe una tendencia histórica a la reducción de la harina de pescado incluida en dietas para acuicultura, mientras que también las tasas de conversión alimenticia han tendido a reducirse (Olsen & Hasan 2012). Los costos estimados de producción para generar un kilogramo de biomasa microbiana son altamente variables, pero en general también se ha experimentado una reducción progresiva tanto en los costos de producción como en los precios de venta gracias a nuevas tecnologías implementadas. Loo *et al.* (2013) han reportado que los costos estimados de producción de microalgas y levaduras para operaciones de larvicultivo se encontraban previamente en un amplio rango de 46 a 600 USD por kilogramo de biomasa seca. Sin embargo, métodos recientes de producción para bacterias y levaduras han disminuido significativamente estos números y se encuentran actualmente en un rango de 1 a 79 USD por kilogramo de biomasa seca (Rosenberry 2011; Pongpet *et al.* 2015). Los principales costos de producción están representados por la obtención de sustratos de cultivo y la energía requerida para las operaciones de cultivo, cosecha, secado, etc. Por lo tanto, técnicas de producción menos

onerosas se han enfocado en el uso de sustratos no convencionales para producir biomasa microbiana en una forma sustentable y económica. Por ejemplo, Lee & Kim (2001) demostraron que los costos de producción de la levadura torula *Candida utilis* puede reducirse hasta 1.08 - 2.76 USD kg usando melaza en lugar de medios de cultivo complejos.

Métodos alternativos para la producción de levaduras y bacterias a partir del uso de sustratos representados por desechos de las industrias agrícolas y de los alimentos, se encuentran actualmente en etapa experimental o piloto comercial (Dantas *et al.* 2016; Nouska *et al.* 2015). No obstante, otros procesos ya han sido estandarizados, escalados y patentados. Algunos de estos productos serán exclusivamente orientados al sector de la nutrición animal y la acuicultura (Glencross *et al.* 2014; WEF 2015; BFD 2015). Frecuentemente, el uso de la biomasa microbiana recién cosechada requiere tratamientos post-cosecha aplicados para concentrar la cantidad de proteína, para mejorar la digestibilidad o para satisfacer aspectos de inocuidad. Todos estos procesos tienden a aumentar el precio por unidad de biomasa microbiana producida. Vizcaíno *et al.* (2014) realizaron un estudio para utilizar la microalga *Scenedesmus almeriensis* como un ingrediente de reemplazo de harina de pescado en dietas para dorada, pero se considera que los precios de producción deben disminuir ya que, por ahora, el precio (7 a 14 USD Kg) limita el uso de esta biomasa a gran escala. Acién *et al.* (2012) demostraron que los costos de producción de una instalación real para la producción de microalgas de esta misma especie pueden reducirse de 75 a 14 USD kg mediante la simplificación de las tecnologías de producción y el escalamiento de la producción (en particular los foto-biorreactores). De la misma forma, Norsker *et al.* (2011) calcularon los costos de producción para generar biomasa de microalgas en tres diferentes sistemas operando a escala comercial: estanques abiertos, foto-biorreactores tubulars horizontales y foto-biorreactores en panel. Para los tres sistemas, los costos de producción para un kilogramo fueron 5.50, 4.45 y 6.62 USD, respectivamente. Los elementos más costosos en el proceso de producción estuvieron representados por el establecimiento de condiciones de irradiación óptima, el mezclado de fluídos, la procuración de alta eficiencia fotosintética, los medios de cultivo y el dióxido de carbono suplementario. Después de optimizar la producción respecto a cada uno de estos

factores, el precio resultante de la biomasa microalgal fue de 0.77 USD kg. Los autores declaran que a este nivel de precio, las microalgas denotarían ser un insumo promisorio como alimento o suplemento nutricional, así como una fuente rentable de biocombustibles y de compuestos orgánicos específicos.

Retos actuales y futuros

Las técnicas de producción mejoradas y la aplicación de nuevos métodos para aumentar el valor nutricional de los productos microbianos finales son dos de los aspectos que determinarán que tan rápido la biomasa microbiana será usada de forma común como parte de los alimentos acuícolas. Los métodos industriales más avanzados para generar biomasa microbiana corresponden a aquellos aplicados a la producción de levaduras. Sin embargo, aún hay varios puntos que mejorar; por ejemplo, los aspectos moleculares relacionados a la adaptación de las levaduras a las adversas condiciones de cultivo, se encuentran poco explorados (Gómez-Pastor *et al.* 2011). El mejoramiento de cada una de las etapas de producción aportará una mayor producción de biomasa y productos finales derivados de ésta. Básicamente, cualquier modificación de la biomasa microbiana en las etapas de postproducción, tiende a incrementar los costos de producción; por lo tanto, es requerida investigación adicional para evaluar las características nutricionales de la biomasa microbiana tratada bajo diversos métodos y comparada contra la biomasa intacta. Actualmente se aplican diversos métodos para mejorar las propiedades nutricionales de los productos finales destinados a ciertos organismos consumidores. La pared celular de los microorganismos puede imponer restricciones para estos animales. Por ejemplo, la pared celular representa 10% de la biomasa microalgal en peso seco y representa un problema para la completa digestión o utilización de esta biomasa para humanos y para organismos no-rumiantes. Procedimientos innovadores orientados a romper la pared celular de los microorganismos son actualmente necesarios para incrementar la biodisponibilidad de los nutrientes. Resultados de varios estudios indican que al remover las paredes celulares o al extraer productos específicos, se obtienen claros beneficios nutricionales e inmunológicos, al comparar los efectos con la biomasa microbiana sin tratar. Rumsey *et al.* (1991) han reportado que después de romper las paredes celulares de las levaduras, se obtuvo un

incremento de 20% en la absorción de nitrógeno en trucha arcoiris (*Oncorhynchus mykiss*). Estudios recientes realizados en peces y crustáceos han demostrado que el crecimiento, la utilización de nutrientes, la digestibilidad de las dietas y las respuestas inmunológicas se han mejorado significativamente mediante la inclusión dietaria de extractos de levaduras (Biswas *et al.* 2012; Berto *et al.* 2015; Zhao *et al.* 2017).

Además de las diversas técnicas empleadas para romper las paredes celulares, existe otro rango de metodologías aplicadas para remover el típicamente alto contenido de ácidos nucleicos presentes en la biomasa microbiana (Skrede *et al.* 2009; Oliveira & Oliva-Neto 2011). Por ejemplo, la levadura contiene entre 6 y 15% de ácidos nucleicos comparado al 2% en los productos cárnicos (Arora *et al.* 1991). Los animales terrestres monogástricos son incapaces de tolerar altos niveles de nucleótidos dietarios (>4 g de purinas y pirimidinas por día) debido a que se producen altas concentraciones de ácido úrico en plasma, además de la generación de gota a partir del metabolismo de las purinas. Adicionalmente, existen estudios que reportan efectos adversos de los nucleótidos sobre el metabolismo de otros nutrientes (Rumsey *et al.* 1992; Devresse 2000). En nutrición humana, un contenido de ácidos nucleicos de 1% en los alimentos ingeridos es considerado como el límite superior (Weissermel & Arpe 2003). De forma interesante, los peces parecen capaces de tolerar niveles de ácidos nucleicos dietarios significativamente mayores gracias a la presencia de la enzima hepática uricasa (urato oxidasa) (Kinsella *et al.* 1985; Oliva-Teles & Goncalves 2001). Do Huu *et al.* (2012) determinaron la semi-escencialidad de los nucleótidos en la dieta del camarón tigre y definieron un rango dietario óptimo de entre 0.48 y 0.56 %. Estudios adicionales en crustáceos y en otros organismos acuáticos son actualmente requeridos para definir los efectos de diferentes niveles de inclusión dietaria de los nucleótidos al ser administrados como inmuno-estimulantes. Tales estudios también arrojarían nueva información sobre los límites superiores de inclusión. Existen otras aproximaciones para manipular el valor nutricional de la biomasa microbiana. Becker (2007) reportó en un estudio sobre biomasa de microalgas, el efecto de diferentes métodos de tratamiento post-cosecha (secado por aire, secado en tambor, secado al sol) sobre diferentes cualidades nutricionales de la biomasa obtenida. En el caso de la producción de biomasa bacteriana, Loo *et al.* (2013) indican en un estudio que el perfil nutricional de una

biomasa bacteriana deficiente en lípidos puede ser mejorado al cultivar las bacterias en sustratos alternativos derivados de las industrias de los alimentos. Sustratos alternativos y sustentables que también han sido probados para generar biomasa bacteriana, incluyen el uso de desechos agrícolas, efluentes de procesadoras de aceite de palma y los lodos activados de las plantas de tratamiento de agua.

Conclusiones

La producción futura de biomasa microbiana y los diversos ingredientes derivados de ésta, se incrementará significativamente no solo debido al desarrollo de técnicas mejoradas de producción, sino también en respuesta a una creciente demanda y a una diversificación de las aplicaciones en la nutrición acuícola. Como se ha observado frecuentemente con otros insumos, altas producciones pueden conllevar a menores precios. Sin embargo, el precio de la biomasa microbiana será significativamente dependiente de la reducción de los costos de producción. En este contexto, el uso de sustratos alternativos y menos onerosos, representa uno de los principales aspectos que conllevaran a precios más competitivos. Por ejemplo, Gómez-Pastor *et al.* (2011) consideran que la producción industrial de levadura se beneficiaría a partir de nueva investigación orientada a las eficiencias fermentativas y a la identificación de elementos causantes de estrés en la producción de levadura, algunos de estos aspectos también aplican a la producción de microalgas bajo condiciones heterotróficas. El futuro de la producción de biomasa microbiana para su uso en alimentos acuícolas también dependerá del mejoramiento de las técnicas de producción para lograr un mejor valor nutricional de los productos finales, todo esto a través de innovaciones creativas. La aplicación de técnicas genéticas convencionales y tecnologías de ADN, permitirán la continuación del desarrollo adaptativo de los microorganismos cultivados (Poulose & Bright Singh 2014). Por otro lado, diversas técnicas de evaluación nutricional y la disponibilidad de instrumentación analítica más sensible, facilitarán la investigación nutricional acerca de los efectos de la biomasa microbiana sobre la fisiología de los organismos acuáticos.

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Taurine synthesis in teleost-importance of cysteamine pathway

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Abstract

Taurine plays various roles in animals such as growth promotion, osmoregulation, bile acid conjugation, neurotransmission, cardiac muscle contraction, antioxidant activity, and reproduction. Taurine is one of the essential nutrients for marine fish larvae and in fishes which lack endogenous taurine production. Taurine is synthesized from methionine via cysteine. Cysteine is converted to cysteine sulfinic acid by activity of cysteine dioxygenase (CDO) and cysteine sulfinic acid is converted into hypotaurine by cysteine sulfinic acid decarboxylase (CSD) in CSD pathway which is considered to be a major taurine production pathway in fish. Hypotaurine is finally converted into taurine by auto-oxidation. In addition to CSD pathway, there is two other taurine synthetic pathways are known: cysteic acid pathway where cysteine is oxidized into cysteic acid, and it is directly converted into taurine by cysteic acid decarboxylase (CAD) activity and cysteamine pathway where cysteine is converted into cysteamine and it is converted into hypotaurine by cysteamine dioxygenase (ADO). However, detail on taurine production by these two pathways is not understood.

Common carp is widely cultured in the world and world production of cypriniforms is highest among food fish species. Rainbow trout is known to have sufficient CSD activity to produce taurine via methionine. In contrast, it was reported that CSD activity in common carp is about half of that reported in rainbow trout. However, common carp did not show growth retardation when it was fed taurine deficient diet. These observations led

hypothesis that common carp is able to produce sufficient amount of taurine beside the CSD pathway. The purpose of the present study is to investigate effect of dietary supplementation of cysteine, cysteamine, methionine, and taurine on the growth, sulfur amino acid content, and gene expression of taurine synthesizing enzymes.

Eight different diets supplemented with taurine, methionine, cysteine, and cysteamine were fed to the juvenile common carps for 30 days. For control, a diet without supplying sulfur amino acid was fed. Feeding diets supplemented sulfur amino acid resulted in better survival, growth, feed conversion ratio, and protein efficiency ratio except treatments supplemented with cysteamine. It was observed that the supplementation of dietary cysteamine caused growth retardation, myopathy, and body deformity in common carp. All sulfur amino acids increased taurine deposition in the carcass and 1.5% cysteamine increased taurine deposition by 1.8 and 5.5 times higher than those of the methionine and cysteine treatments. CDO was tended to be down-regulated by cysteine and low dose of taurine but up-regulated by a high dose of cysteamine. It was observed that CSD was down-regulated by sulfur amino acids. ADO was down-regulated by methionine, cysteine and low dose of taurine but up-regulated by cysteamine.

These results suggest that CSD pathway plays a role in taurine synthesis and cysteamine pathway is another major taurine synthesizing pathway in common carp.

Keywords: Taurine, Synthesis, Teleosts

Introduction

World population increase and food crisis has been warned to occur in near future. Contrast to stable production in capture fisheries without increase, world aquaculture production has increased about twice in the last decades and occupies 47% of total fishery production (FAO 2016). Fish requires relatively higher dietary protein than terrestrial animals and fishmeal is the main protein source in fish feed. Major fishmeal producer in the world is Chile and Peru. However, in order to save natural fish resources, development of fish meal free diet has been a subject for achieving sustainable aquaculture industry for next generation. Currently, fishmeal free diet can be used for laboratory experiment but supplementation of important nutrients such as taurine is essential.

Taurine plays various roles in growth, vision, reproduction, neurotransmission, osmoregulation, and antioxidant mechanism in fish (Takeuchi 2014). Although it was reported low taurine biosynthetic ability in marine carnivorous fish species, we have successfully isolated cysteine sulfonate decarboxylase, which is considered to be the rate limiting enzyme of taurine synthesis, from marine fishes such as red sea bream *Pagrus major* and yellowtail *Seriola quinqueradiata* (Haga *et al.* 2015). Detailed analysis of modulation of gene expression of CSD genes could lead development of technique to enhance taurine biosynthetic ability in fish. This paper reviews recent finding on taurine function in fish. Although detailed description on taurine transporter was not made in this paper, readers should refer appropriate reviews that published previously (Warskulat *et al.* 2007; Han *et al.* 2006; Xu *et al.* 2015).

1. Chemical aspect of taurine

IUPAC name of taurine is 2-ethanesulfonic acid and its molecular weight is 125.12. Taurine crystal is white spindle shape. Definition of amino acid is that the chemical has to have carboxylic acid as well as amino group in its molecule. Taurine molecule has sulfonic acid instead of carboxylic acid. So taurine is amino acid related compound but not true amino acid. Taurine is rich in fish and shellfish, aquatic reptile, cardiac muscle of animals, colostrum of humans (Jacobsen and Smith 1968; Murakoshi and Hatanaka 1977; Ozawa *et al.* 1984; Suyama *et al.*, 1979). Taurine is not included in higher terrestrial plants (Jacobsen and Smith 1968). Taurine has been isolated from bovine liver for human use.

2. Taurine content in aquatic organisms and tissue distribution

Aquatic organisms contains high amount of taurine and its related compounds (Table 1). Ozawa *et al.* (1984) examined taurine content in muscle of more than 50 species and found 11-356 mg/g taurine. Ohyama *et al.* (1991) also examined more than 11 fish species and 10 cephalopod, shellfish, crustacean and algae and found relatively high in muscle of tilefish, barracuda and Japanese anchovy (256-373mg/100g), and around 100-200 mg/ 100 g in other fish species. In addition, 182-1232 g/100mg, 43-75mg/100g and 21-1380mg/100g of taurine was recorded in cephalopod, shrimp, and shellfish respectively. Hiraoka *et al.* (2011) also examined taurine content in 26 fish species and found 15-1265 mg/100 g in tissue and high taurine reported in dark muscles of tuna and amberjacks (451-1265mg/100g) as well as in cephalopod (497-1031mg/100g). Taurine abundantly presents in liver (hepatopancreas),

kidney, dark muscle and gonad (Murakoshi and Hatanaka 1977; Ozawa *et al.* 1984; Sakaguchi and Murata 1987; Suyama *et al.*, 1979). Relatively high level of taurine was also detected in muscle of freshwater turtle (88-134mg/100g) (Suyama *et al.*, 1979). However, it was reported that elevation of taurine content in tuna meat after 72h during cold storage (Shiraita *et al.* 2012). So readers should keep in mind that reported taurine content in fish can be affected by changes after storage. Among prey organisms used for juvenile production in hatchery, taurine was rarely found in rotifer (80-180 mg/100 g) but considerable level of taurine was reported in *Artemia* (400-800 mg/ 100 g) with difference between strains (*A. franciscana*; 463 mg/100g, *A. tibetiana* 632 mg/100mg) (Kurihara 2008; Takeuchi 2014). Although large variation was found in taurine levels in wild zooplanktons depending on nutritional condition, 800-1200 mg/100 g of taurine was reported (Matsunari *et al.* 2003). Highest level of taurine was found in mysids among feed items used in aquaculture (2900mg/100g, Seikai *et al.* 1997). Red algae is known as rich in taurine derivatives; taurine, N-methyltaurine, N, N-dimethyltaurine, and N, N, N-trimethyltaurine were reported (Impellizzeri *et al.* 1975; Lindberg 1955; Ito 1965). D-Glycerotaurine was isolated from suginori *Gigartina leptorhynchos* and okitsunori *Gymnogongrus flabelliformis*. D-cysteinolic acid was found in algae *Polysiphonia fastigata*, sardine *Sardinopus melanostica* (Satake *et al.*, 1987), and starfish *Asteria Pectinifera* (Yoneda and Yoshimura, 1965).

Taurine content does not decrease in early embryogenesis but quickly decreases during ontogeny of Japanese flounder and yellowtail after hatching (Matsunari *et al.* 2003; Takahashi *et al.* 2005; Takeuchi *et al.* 2001). Comparing taurine content in wild and cultured yellowtail juveniles suggest that considerably lower taurine content was found in cultured

yellowtail than wild juvenile and suggested that taurine deficiency in cultured yellowtail juveniles (Matsunari *et al.* 2003). Higher taurine content in wild fish than cultured fish was also reported in Japanese flounder, red sea bream, and four-spine sculpin (Kim *et al.* 2000; Morishita *et al.* 1989; Iwatani *et al.* 2012). This kind of low taurine content in cultured fish was able to be improved by feeding zooplanktons enriched with taurine (Takeuchi 2014) and several taurine enrichment products have been commercially available in Japan. It was suggested that taurine content in fish is not only affected by dietary taurine intake (Matsunari *et al.* 2008; Takahashi *et al.* 2005) but also dietary protein in rainbow trout (Yamamoto *et al.* 2000).

3. Taurine synthetic pathway in animals

Taurine was synthesized from methionine and cysteine in animal body. Methionine is converted into cystathionine via homocysteine with the aid of cystathionine beta synthetase (CBS). Cystathionine is converted into cysteine by cystathionine gamma lyase. There are three taurine synthetic pathways are proposed; cysteine sulfinic acid decarboxylase (CSD) pathway, cysteamine pathway, and cysteic acid pathway. In CSD pathway, cysteine was converted to cysteine sulfinic acid by cysteine dioxygenase (CDO) and then cysteine sulfonate is decarboxylated to hypotaurine with activity of CSD. Hypotaurine is converted into taurine by auto-oxidation. CDO requires Fe^{2+} for maximum activity of 25000 Da and inactivated with chelate agent such as EDTA. On the other hand, CSD from red sea bream, yellowtail and Japanese flounder possesses NPHK motif in its primary sequence of enzyme protein and suggested to require vitamin B6 for maximum activity (Haga *et al.* 2015; Wang *et al.* 2016).

Hypotaurine is converted into taurine without aid of enzyme activity, rate limiting step of taurine production by CSD pathway is that conversion of cysteine sulfinic acid into hypotaurine. Therefore, CSD activity seems key regulator of taurine production in animal body except for two other taurine synthetic pathways. Cysteine is also important because it has biological activity as a precursor of cysteine sulfinic acid. Cysteine dioxygenase (CDO) is responsible for conversion of cysteine into cysteine sulfinic acid and CDO knockout mice exhibit severe taurine deficient syndrome even if these mice possess CSD activity (Ueki *et al.* 2011). Further, since CDO strongly expresses in placenta of human, although CSD is indispensable for taurine synthesis in humans, it was estimated that CDO primarily determines taurine status in body of mouse and human.

It was suggested that rat CSD not only play a role in decarboxylation of cysteine sulfonate but also decarboxylation of cysteine acid (Jacobsen and Smith 1968). In red sea bream, elevation of supplemental cysteine sulfinic acid under the presence of cysteic acid leads decrease of taurine produced from cysteic acid, suggesting CSD from red sea bream also recognize and convert cysteic acid as well as cysteine sulfonic acid as a substrate (Goto *et al.* 2003). In contrast, glutamic acid decarboxylase-like enzyme 1 (GADL1) which is only found in amphibian, avian, and mammals is known to function not only in decarboxylation of glutamic acid but also taurine synthesis (Liu *et al.* 2012; Winge *et al.* 2015). Hence, it was reported that mammalian GADL1 is responsible for conversion of aspartic acid into β -alanine and it also convert cysteine sulfinic acid and cysteic acid into hypotaurine and taurine, respectively (Liu *et al.* 2012). We isolated full length primary sequence of cysteine sulfinic acid decarboxylase from red sea bream and yellowtail and found that high expression was

commonly found in liver and pylorus in red sea bream, Japanese seabass, spotted halibut and yellowtail, suggesting CSD plays a role in taurine production in these tissues in fishes. Wang *et al* (2016) examined CDO and CSD in Japanese flounder and rainbow trout. They found that CDO expression was downregulated by taurine supplementation in rainbow trout, suggesting CDO plays a role in maintain taurine level in fish body (Wang *et al.* 2016). On the other hand, CSD but not CDO expression was downregulated in Japanese flounder (Wang *et al.* 2016). In addition, zebrafish CSD was regulated by dietary taurine level (Chang *et al.* 2013). These findings suggest that rate limiting step taurine production by CSD pathway is different depending on fish species. In turbot *Psetta maxima*, cysteine and methionine elevated level of CDO transcript (Wang *et al.* 2014). Common carp was reported to possess CDO1 and 2 and CSD, yeast hybrid expressed fusion protein of CDO1 or 2 with CSD resulted in higher taurine production potency was seen in CDO1/CSD fusion protein, suggesting CDO1 has more higher catalytic activity (Honjoh *et al.* 2009).

Taurine transporter 1-3 play a role in taurine intake in cell and presence of TauT3 paralog in fish was suggested. Mobilization of taurine by GABA transporter was also suggested and some papers suggested that GABA transporter is more important than TauT in taurine intake in cell (Liu *et al.* 1993; Zou *et al.* 2012). However, there is few report on taurine transport by GABA transporter in fish.

4. Function of taurine in fish

Taurine plays a role in bile acid conjugation and excretion, lipid absorption, skin development, vision, swimming and feeding activity, reproduction, growth and development of early stage, stress response, and taste of fillet in fish.

Taurine conjugates bile salt. Glycine also does but taurine conjugated bile salt has more potent in solubility in water. Selectivity of organic acid for bile salt conjugation is diverse in teleostean species (Hagey *et al.* 2010). In carp, cyprinol sulfate, causing food poisoning toxin for carp consumer, is a main bile acid conjugate and occupies more than 94% in total bile acid (Yeh and Hwang 2001). On the other hand, taurocholic and taurodeoxycholic acids are the major bile acid constituents in Japanese flounder (Goto *et al.* 1996). Bile pigment is originated from hemoglobin in blood and blood cell has shortest biological half-life in cell of animal body. Therefore, degraded hemoglobin gives rise bililvin and bilibergin that have to be conjugated by taurine for excretion. Because water solubility of these pigments is conferred by conjugation with taurine, taurine deficiency causes abnormal accumulation of pigments and malfunction of liver and eventually led mortality. The fact that fish highly depends on taurine for bile conjugation has higher risk of taurine deficiency since they has to always consume a certain amount of taurine for maintenance of normal function of liver. Conversely, taurine deficiency seems to less occur in fishes that are able to use other organic acid for bile conjugation even when they fed low taurine diet such as plant protein based diet. Dietary intake of taurine is controlled by taurine level in a diet as well as food intake of fish. Therefore, risk of taurine deficiency is higher in winter when feed intake decreases. In fish farming site, water temperature and feed intake should be carefully observed when one

consider risk of taurine deficiency. Red sea bream requires dietary taurine when fed low fishmeal diet but its requirement in the fish is lower than yellowtail (Takagi *et al.* 2011).

Blighter skin color was reported in red sea bream fed casein based diet supplemented with taurine (Takeuchi 2014). It was reported that taurine supplementation on fishmeal based diet enhanced skin thickness and improved occurrence of scale detaching at harvesting fish (Kato *et al.* 2012; 2014). This observation was also reproduced when fish was fed moist pellet formulated with low level of fishmeal (30%) and skin thickness became 80 to 120 μ m by dietary taurine supplementation at high water temperature. However, they failed to observe effect of taurine on skin thickness at the season with low water temperature. It was probably because lower level of feed intake in cold season compared to high water season. Taurine is one of the popular components of hair treatment detergent for humans and hair growth promotion was reported by taurine supplementation. Taurine seems to promote growth of skin and its appendages in vertebrates.

Improvement of reproductive performance and larger size eggs were reported in yellowtail (Matunari *et al.* 2008). Similar improvement of reproductive performance and enhanced survival after hatching were reported in tilapia (Al-Feky *et al.* 2016). Higher taurine content in fertilized egg than unfertilized egg was found in swimming crab and elevation of taurine content was observed before hatching (Paneflorida 2004). In addition, comparing 20,000 transcripts from bloodstock with better reproductive performance (survival was less than 84% at seven days post fertilization) and worse bloodstock (survival was less than 6% at seven days post-fertilization) revealed that one of the transcripts with significant difference was CSE which is cysteine synthetic enzyme gene (Rise *et al.* 2014). These results suggest

that taurine and its precursor are important for reproduction success and early survival of embryos.

Improvement of feeding behavior was reported in red sea bream juveniles fed taurine supplemented casein based diet (Matsunari *et al.* 2008). Faster swimming speed toward tank bottom was observed immediately after completion of feeding in flounder juveniles (Kim *et al.* 2005). In addition, improvement of survival after releasing in wild environment was observed in Japanese flounder fed taurine supplemented diet (Morita *et al.* 2011). Taurine is one of the important energy source for early stages of fish (Ronnestad *et al.* 2003). In mammals, taurine occupies more than 50% of total free amino acid in eye ball and cardiac tissue. More than 70% of total free amino acid was reported to be occupied by taurine in heart ventricle in Pacific bluefin tuna (Ishihara *et al.* 2013). Reduction of plasma taurine and cysteine was reported in rat after 8 weeks of exercise (Gaume *et al.* 2005). Taurine deficiency in cardiac and skeletal muscle in fish could hinder normal function of these locomotive organs resulted in lower swimming performance of fish.

Importance of taurine in stress response has been suggested by studies in mammals. Taurine plays a role in removal of reactive oxygen species. When gilthead seabream *Sparus aurata* was exposed to confinement stress, 202 gene transcripts responded; among them, CBS and CDO responded in phase 1 when energy metabolism was reconstructed for acute response, and CSE responded in phase 3 when reconstruction of cellular homeostasis for selective classification and destruction of reactive oxygen species (Calduch-Giner *et al.* 2010). CBS expresses in central nervous system of chinook salmon, common carp, and zebrafish as a source of H₂S (Pushchina *et al.* 2011; Porteus *et al.* 2014), Cells with H₂S suggested to be involved in enhancement of respiratory volume as a sensor of low oxygen condition

(Pushchina *et al.* 2011). These findings suggest that synthesis of taurine precursor enhanced at stressed condition and serves as a source of H₂S which act as signaling molecule.

Taurine is also suggested to affect taste of fillet of fish. When panellers sampled fillet of yellowtail *S. quinquerediata* fed a diet supplemented taurine for 18 weeks, there was no difference in taste of fillet from fish fed taurine supplemented diet and a diet formulated with 60% fishmeal but lower score was obtained for fillet from the abdominal part of fish fed low fish meal diet than taurine supplemented diet (Khaoian *et al.* 2014). There is also no effect on selection of choice “like” or “dislike” when the fillet was tasted with or without soy source (Khaoian *et al.* 2014). In addition, similar trial was also made in yearling greater amberjack *Seriola dumerili*. Extruded pellet with a low fishmeal diet was formulated with soy protein concentrate, soybean meal, and corn gluten meal. Fillet samples was tested after feeding the low fishmeal diet (10-30%) with or without 0.2-0.4% taurine for 114 days and highest score was obtained in fillet from fish fed a low fishmeal diet with 0.4% taurine was recorded (Maeno *et al.* 2012). Collectively, these reports suggest that taurine supplementation could affect on taste of fillet of fish.

Although there is few report on taurine function in invertebrates, feeding rotifers containing 2.83mg/g taurine to Pacific whiteleg shrimp *Litopenaeus vannamei* enhanced developmental morphological changes of shrimp larvae as well as improved survival (Jusadi *et al.* 2011).

As we described, taurine has diverse array of biological activity and affect normal growth, development, function, and fillet quality of fish. Therefore, it has been approved to use as supplement of fish feed in 2002 in Japan. Because FDA has also approved to use of synthetic taurine in fish feed in US on March 2017, it is expected that practical use of feed with synthetic taurine will be tested for various fish species at large scale production level.

Considering basic science, fish species can be a good model organism to study function of taurine because of its abundance and diverse metabolic ability. Therefore, detailed study on taurine synthetic ability and its control mechanism will lead future achievement such as development of high taurine strain of food fish by breeding program or genome editing technique, etc.

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Development of Antibiofilm Biosurfactants from Marine Bacteria Against Shrimp *Vibrio* pathogens

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Abstract

Vibrio disease is being described as a major bacterial disease obviously known as penaeid bacterial septicaemia, penaeid Vibriosis, luminescent Vibriosis or red leg diseases. Signs of *Vibrio* disease include lethargy, tissue and appendage necrosis, slow growth, slow larval metamorphosis, body malformation, bioluminescence in shrimp particularly produced in flocc systems, muscle opacity, melanization, empty midgut and anorexia. In Asia, *V. alginolyticus* and *V. harveyi* were considered as the most significant pathogens in the grow-out ponds of giant black tiger shrimp *Penaeus monodon*. Survival and pathogenicity of *Vibrio* was associated with the biofilm formation and quorum sensing. Therefore, disruption of biofilm formation and/or quorum sensing would be an effective management strategy in aquatic systems instead of killing the pathogens which obviously leads to the development of resistant strains. Biosurfactants are surface active smart biomolecules showed strong antibiofilm activity against *Vibrio* pathogens. In this report, biofilm producing *Vibrio* pathogens include *V. harveyi* VB1, *V. alginolyticus* VB2, *V. vulnificus* VB3, *V. fischeri* VB4, *V. parahaemolyticus* VB5 and *Photobacterium damsela* VB6 were isolated from the moribund shrimp samples collected from farms located southeast coast of India. Based on their surface-active properties, we hypothesized that biosurfactants could disrupt biofilms of *Vibrio* pathogens. To test the hypothesis, we examined the effects of the lipopeptides extracted from marine bacteria MSI-A 07 and MSI-A 08, on the biofilm-forming capacity of biofilm infection causing pathogenic *Vibrio* spp. (*V. harveyi* VB1, *V. alginolyticus* VB2, *V. vulnificus* VB3, *V. fischeri* VB4, *V. parahaemolyticus* VB5 and *Photobacterium damsela* VB6). The both lipopeptide biosurfactants potentially disrupted biofilm formation under dynamic conditions. The biofilm disruption potential of the lipopeptide biosurfactants was consistent against all shrimp pathogens. Based on this finding, biosurfactant incorporated feed can be formulated to contain *Vibrio* outbreaks in shrimp aquaculture.

Key words: Biosurfactants, vibriosis, shrimp aquaculture, biofilm disruption

Introduction

Aquaculture is the quickest developing sustenance part all around and it was quickly venturing into multibillion dollar industry. Be that as it may, by and by, the real inconvenience confronted by the aquaculture business worldwide is infections caused because of different biological and non-biological operators. The biological agents bacteria, virus and fungi were accepted to be the reason of extreme monetary misfortune in the incubation centers and develop out lakes in all aquaculture creating nations (Ruangpan and Kitao, 1991). Among the gatherings of microorganisms that reason serious misfortunes in shrimp culture, the best known are bacteria as a result of the stunning financial impacts they have on influenced ranches (Lightner, 1996; Karunasagar *et al.* 1994).

Pathogenic Vibrios are one of the significant wellsprings of shrimp sickness as a result of their nearby relationship with low survival rates in hatcheries and develop out lakes. In many shrimp cultivating areas, infections credited to *Vibrio* spp. are viewed as the most regular and vital irresistible issues (Ruangpan & Kitao, 1991; Sung *et al.* 2001). Vibrios can ability to grow as biofilm with resistance to disinfectants and antibiotics that cause a variety of shrimp disease in hatcheries and grow- out ponds (Karunasagar., 1994, 1996; Alvarez *et al.* 1998). In 1996, Karunasagar *et al.* revealed the antibiotic resistant *Vibrio harveyi* held on in the larval tanks of a shrimp hatchery, most presumably as biofilm bacteria and consequently not effectively expelled by sanitizer treatment.

Vibrio biofilm

In most environmental specialties, Vibrios are developed on regular or artificial surfaces as single or multispecies groups known as biofilms. A biofilm is a sessile microbial group comprising of cells that are irreversibly joined to a substratum and installed in an extracellular polymeric framework (Donlan & Costerton, 2002). The vast majority of the investigations show that biofilms are a steady point in a natural cycle that starts with the vehicle and connection of the bacterium to surfaces. After the underlying connection, colonization of a surface is interceded by the development and development of appended microbes. Surface colonization at that point prompts

the development of microcolonies, which are frequently encompassed by extrapolymeric substances. Assist development of bacteria and proceeded with creation of exopolysaccharide prompt the advancement of develop biofilm structures portrayed by columns and channels. It has been demonstrated that advancement of these structures relies upon biomass development rate, twitching motility, signalling molecules, and production of exopolysaccharide (O'Toole *et al.* 1998; Costerton *et al.* 1995; and Parsek & Fuqua, 2004). As per the O'Toole. (1998) the physiology, cell surfaces, imperviousness to natural put-down, and different properties of biofilm cells are notably not the same as their planktonic partners. Biofilm development rises as an essential component for microbial survival in nature. Vibrios are ubiquitous in situations mostly amphibian environments. The biofilm framing limit of *V. cholerae* is all around archived, both in common environments and under research center conditions (Faruque *et al.* 2006; Watnick and Kolter, 1999; Yildiz & Schoolnik, 1999). A few research discoveries uncover the significance of biofilms in survival, harmfulness, and stress resistance components of *Vibrio* spp. (Watnick & Kolter 1999; Watnick *et al.* 2001; Zhu & MeKalanos, 2003; Faruque *et al.* 2006; You *et al.* 2007; Yildiz & Visick., 2009). With this standpoint the present investigation was intended to assess the biofilm framing capability of pathogenic *Vibrio* spp., related with shrimp malady.

Vibrio disease is depicted as Vibriosis or bacterial infection, penaeid bacterial septicaemia, penaeid Vibriosis, luminescent Vibriosis or red leg maladies and is globally circulated. Indications of *Vibrio* ailment incorporate laziness, tissue and extremity putrefaction, moderate development, moderate larval transformation, body deformity, bolitas negricans, bioluminescence, muscle mistiness, melanization, purge midgut and anorexia (Karunasager *et al.* 1994; Lightner and Redman, 1994; Smith, 2000). In Asia, among the pathogenic *Vibrio* gathering, 11 species were accounted for from the shrimp culture frameworks (Lavilla-Pitogo, 1995). Of these, *V. alginolyticus* and *V. harveyi* are considered as the most huge ones in the develop out lakes of giant black tiger shrimp *Penaeus monodon* in India (Karunasagar *et al.* 1997; Selvin and Lipton, 2003; Manilal *et al.* 2010). The pathogenicity of microbial intruders in the scavenger haemocoel at last lies in the capacity of the life forms to avoid or evade the host resistance systems.

In this examination, hopeless shrimp isolates were morphologically and biochemically portrayed in to six gatherings. Among the six gatherings, the most dynamic biofilm makers were screened. They were named as *V. harveyi* VB1, *V. alginolyticus* VB2, *V. vulnificus* VB3, *V. fischeri* VB4, *V.*

parahaemolyticus VB5 and *Photobacterium damsela* VB6. With respect to antibiotics resistance, all isolates were impervious to chloramphenicol taken after by oxytetracycline. The antibiotic resistance pathogens and amassing of deposits in the shrimp tissue have turned out to be normal in Indian Shrimp ranches (Selvin and Lipton, 2003). Antibiotic resistant *V. harveyi* from tainted larvae showed bring down LD₅₀ esteems for post larval *P. monodon* than *V. harveyi* secludes acquired from sea water (Karunasagar *et al.* 1997). In thinks about by Karunasagar *et al.* (1994, 1996), antibiotic resistant *Vibrio harveyi* persevered in the larval tanks of a shrimp hatchery, most likely as biofilm bacteria and in this way not effortlessly disposed of by sanitizer treatment. These discoveries proposed the potential peril of standard utilization of anti-infection agents in aquaculture and show that they may build the *Vibrio* spp. harmfulness, for example, biofilm development.

All incurable shrimp isolates (*V. harveyi* VB1, *V. alginolyticus* VB2, *V. vulnificus* VB3, *V. fischeri* VB4, *V. para haemolytic us* VB5 and *Photobacterium damsela* VB6) on CRA plate, created black colonies. Slime production assume an essential part in the pathogenesis of contaminations caused by various microorganisms (Alcaráz, 2003; Abdallah *et al.* 2008), and is thought to be a noteworthy harmfulness factor for some *Vibrio alginolyticus* and *Vibrio parahaemolyticus* (Abdallah *et al.* 2008).

Biofilm forming capacity of *Vibrio* pathogens

Quorum sensing assume a key part in the formation of biofilm. Davies *et al.* (1998) distributed the principal ponder that demonstrated a part for majority detecting in the biofilm formation, and propelled a time of dynamic research of cell-to-cell communication in biofilms. McLean *et al.* (1997) have demonstrated that acyl HSL autoinducers are distinguishable in normally happening biofilms, proposing that biofilm groups in nature contain populaces that can experience cell density dependent regulation. In light of the investigation the presence of cell to cell signaling molecule were seen in just 3 biofilm producers (VB3, VB4 and VB5) among the 6 selected isolates (VB1-VB6). In light of the above investigation material and research finding the *Vibrio* spp. detached from the moribund shrimps are recognized as potential biofilm producers *V. harveyi* VB1, *V.*

alginoliticus VB2, *V. vulnificus* VB3, *V. fischeri* VB4, *V. parahaemolyticus* VB5 and *Photobacterium damsela* VB6.

The stereozoom microscope principally used to watch the biofilm amassing in strong surfaces. The biofilm was created on to the microtiter polystyrene plates and it was seen under stereozoom magnifying instrument. The biofilm accumulation on the strong surface was watched and photograph was taken. The accumulation biofilm makers in microtiter plate is fluctuated in light of their surface connection nature. The photograph was shown in Figure 1. The *Vibrio* spp. were delivered distinctive measure of biofilm in the polystyrene microtiter plates.

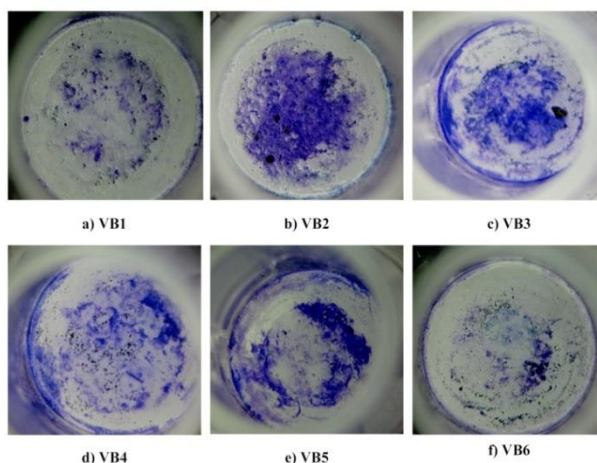


Figure 1. Stereozoom microscope images demonstrating the biofilm forming potential of *Vibrio* spp. (VB1 to VB6).

Biofilm formation is perceived as an imperative destructiveness factor for both opportunistic and true pathogens (O'Toole *et al.* 2000). Bacterial biofilms have a basically intricate and dynamic design and form on numerous abiotic surfaces (plastic, glass, metal and minerals) and biotic (plants, creatures and people) surfaces (Stoodley *et al.* 2002; Hall-Stoodley *et al.* 2004) as single- or various species groups. Biofilm arrangement is a critical component for microbial survival in the earth. Biofilm development is an imperative component for microbial survival in the earth. Biofilm-framing microorganisms are less susceptible to numerous antimicrobial compounds and different biocides. Biofilm formation on medical devices assumes a vital part in the issue of numerous nosocomial and wellbeing related disease and also the development of biofilm favors survival and steadiness of *Vibrio* spp. in the aquatic environment and furthermore inside the host. In light of the above reasons, novel antibiofilm agents are required for the avoidance/control of

pathogenic microbial biofilms on the surfaces and hosts. It was set up that the vast majority of the marine organisms have advanced effective techniques to battle epibiosis. Particularly, marine sponges create particular obstacles to avert biofilm-forming microorganisms (Selvin *et al.* 2010). In any case, it has been estimated that these poisonous hindrances may be delivered by the related microorganisms rather than the host sponge.

Biosurfactants from marine bacteria

Biosurfactants are a heterogeneous group of bioactive amphiphilic particles produced on microbial cell surfaces or extracellularly (Karanth *et al.* 1999). The most potential advantage of microbial surfactants is biodegradability and nontoxicity to common habitats (Banat, 1993). The biomedical significance of biosurfactants was built up because of their antibacterial, antifungal and antiviral properties; hindrance of fibrin clump arrangement; and their anti-biofilm ability against few pathogenic microorganisms (Meylheuc *et al.* 2001, 2006; Singh and Cameotra, 2004; Rodrigues *et al.* 2006). Sponge related marine microorganisms are rising as a potential wellspring of novel biosurfactants (Gandhimathi *et al.* 2009; Kiran *et al.* 2009, 2010). It has been speculated that the antimicrobial fouling process speaks to a substance safeguard of host wipes intervened by the related microscopic organisms. Consequently, the biosurfactants created by the sponge related marine actinobacteria were assessed for the control of pathogenic *Vibrio* spp. biofilms, separated from moribund shrimps.

Biofilm inhibition potential of lipopeptide biosurfactants

In light of their surface-dynamic properties and writing confirm (Kiran *et al.* 2010; Dusane *et al.* 2010), we estimated that glycolipids could influence biofilm arrangement. To test the speculation, we inspected the impacts of the lipopeptide separated from MSI-A 07 and MSI-A 08, on the biofilm-framing limit of biofilm contamination causing pathogenic *Vibrio* spp. (*V. harveyi* VB1, *V. alginolyticus* VB2, *V. vulnificus* VB3, *V. fischeri* VB4, V' VB5 and *Photobacterium damsela* VB6). The both lipopeptide biosurfactants possibly disturbed biofilm development under powerful conditions. The biofilm interruption capability of the lipopeptide biosurfactants was reliable

against all shrimp pathogens. The lipopeptide biosurfactants, specifically MSI-A 07 and MSI-A 08 demonstrated magnificent hindrance against the biofilms of shrimp pathogens (*VB1 to VB6*). The lyophilized lipopeptide biosurfactant were utilized to quantify biofilm inhibitory fixation. To decide the BIC of these two lipopeptide biosurfactants on shrimp *Vibrio* spp., extricates with changed scopes of focuses (10– 50 $\mu\text{g/mL}$) were utilized. Fixation subordinate reduction in biofilm arrangement of test pathogens was gotten upon treatment with the lipopeptide biosurfactants. The biosurfactants got from MSI-A 07 indicated most extreme inhibition of biofilm of 75-80 % at a grouping of 30 $\mu\text{g/mL}$ (Figure 2.1) and the lipopeptide biosurfactant isolated from MSI-A 08 repressed the biofilm development of shrimp pathogens up to 70-75 %, at a focus 40 $\mu\text{g/mL}$ (Figure 2.2). Thus, 30 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$ were fixed as the BIC for MSI-A 07 and MSI-A 08 lipopeptides separately and additionally measures were done at this extract concentration.

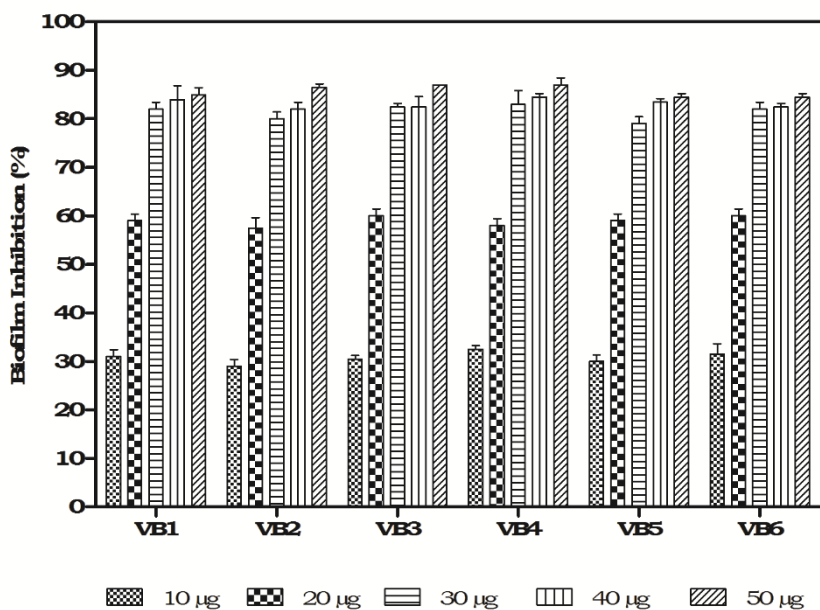


Figure 2. 1. Efficacy of the lipopeptide biosurfactant (MSI-A 07) in the biofilm forming potential of shrimp pathogens.

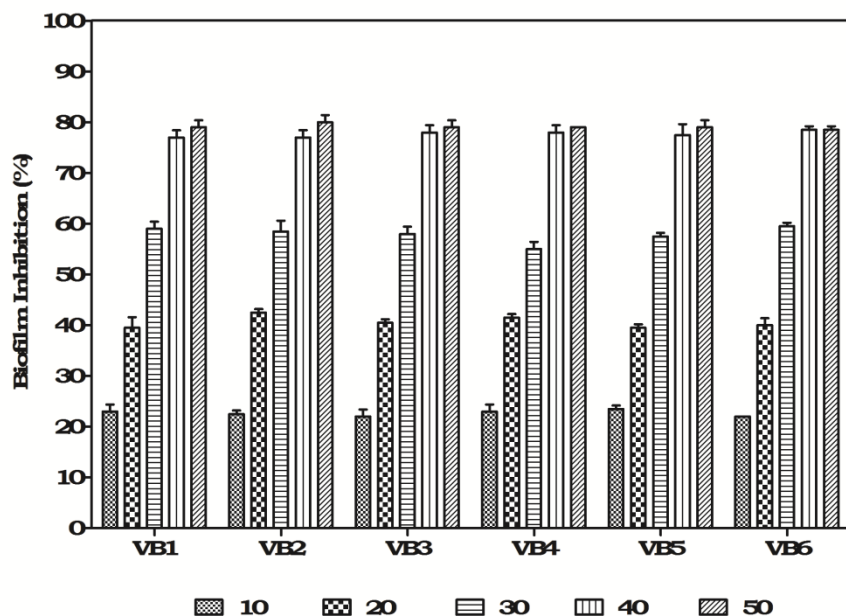


Figure 2.2. Efficacy of the lipopeptide biosurfactant (MSI-A 08) in the biofilm forming potential of shrimp pathogens.

Biofilm disruption potential of lipopeptide biosurfactants

The pictures got from the stage phase contrast microscope uncovered that the lipopeptide biosurfactants extracted from MSIA-07 and MSI-A 08 had potential biofilm disruption. In the cover slip examine, the biofilm disruption was clear and demonstrated a disrupted biofilm under phase contrast microscope (Plate 3.1). These outcomes legibly demonstrate that the lipopeptide biosurfactants disrupts the initial attachment to the surface. one of the important feature of biofilm is the initial attachment. Along these lines, counteractive action of biofilm connection prompts the biofilm disruption. The pictures acquired from light microscope showed that the control slides portrayed all around well-formed biofilm of test pathogens, while, the test pathogens upon treatment with lipopeptide biosurfactants formed poor biofilm development than the control test (Plate 3.3). To decide the outcomes acquired in light microscopy (i.e., breaking down of biofilm structures by biosurfactants), we utilized confocal laser scanning microscopy (CLSM) to additionally illustrate the antibiofilm capability of lipopeptides against biofilms of pathogenic shrimp *Vibrio spp.* (*V. harveyi* VB1, *V. alginolyticus* VB2, *V. vulnificus* VB3, *V. fischeri* VB4, *V.*

parahaemolyticus VB5 and *Photobacterium damsela* VB6) (Plate 3.4). CLSM demonstrated strong adhering capacity of shrimp pathogens, which prompt the improvement of thick biofilm development on glass slide of control samples, while treated samples showed the antibiofilm capability of MSI-A 07 and MSI-A 08 by crumbling the refractory biofilm design of tried pathogens upon treatment. The microtiter plate test likewise demonstrated biofilm disruption capability of MSI-07 and MSI-A 08 lipopeptide biosurfactant on microtiter plates under stereozoom microscopic examination (Plate 3.5).

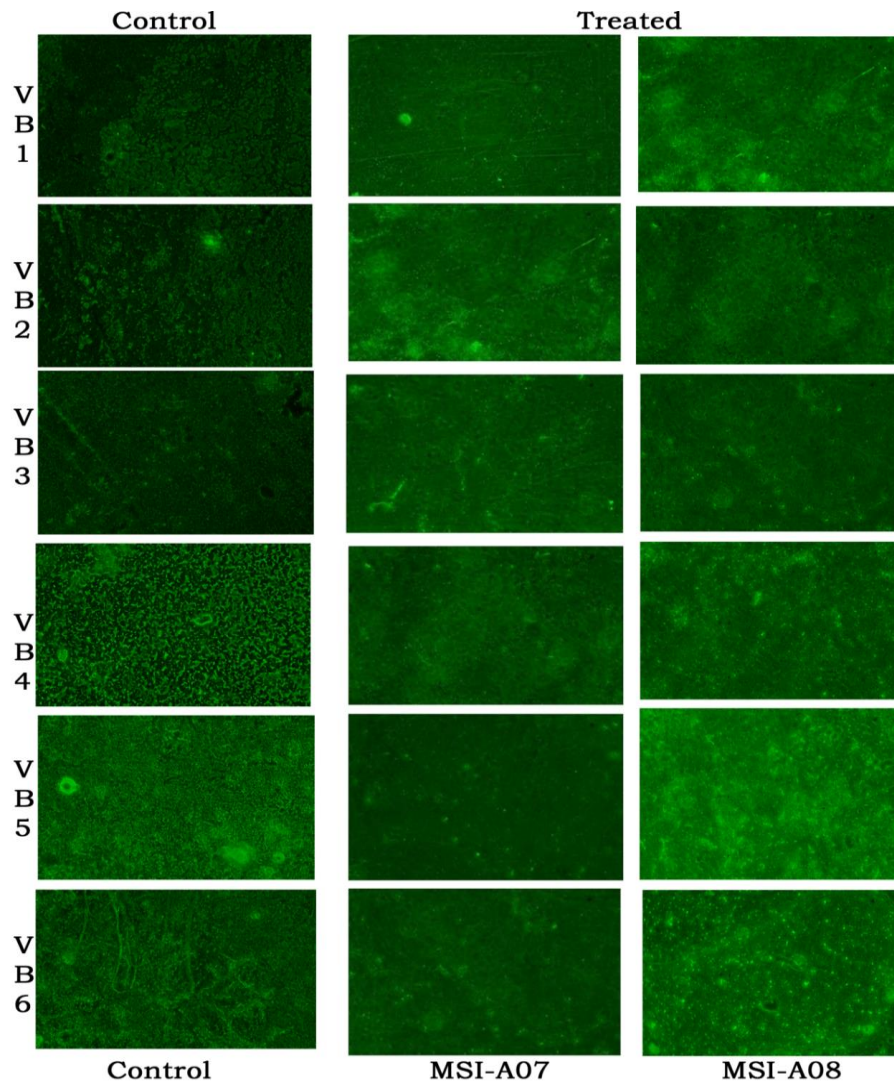


Plate 3.1. Phase contrast microscope images demonstrating the biofilm disruption potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

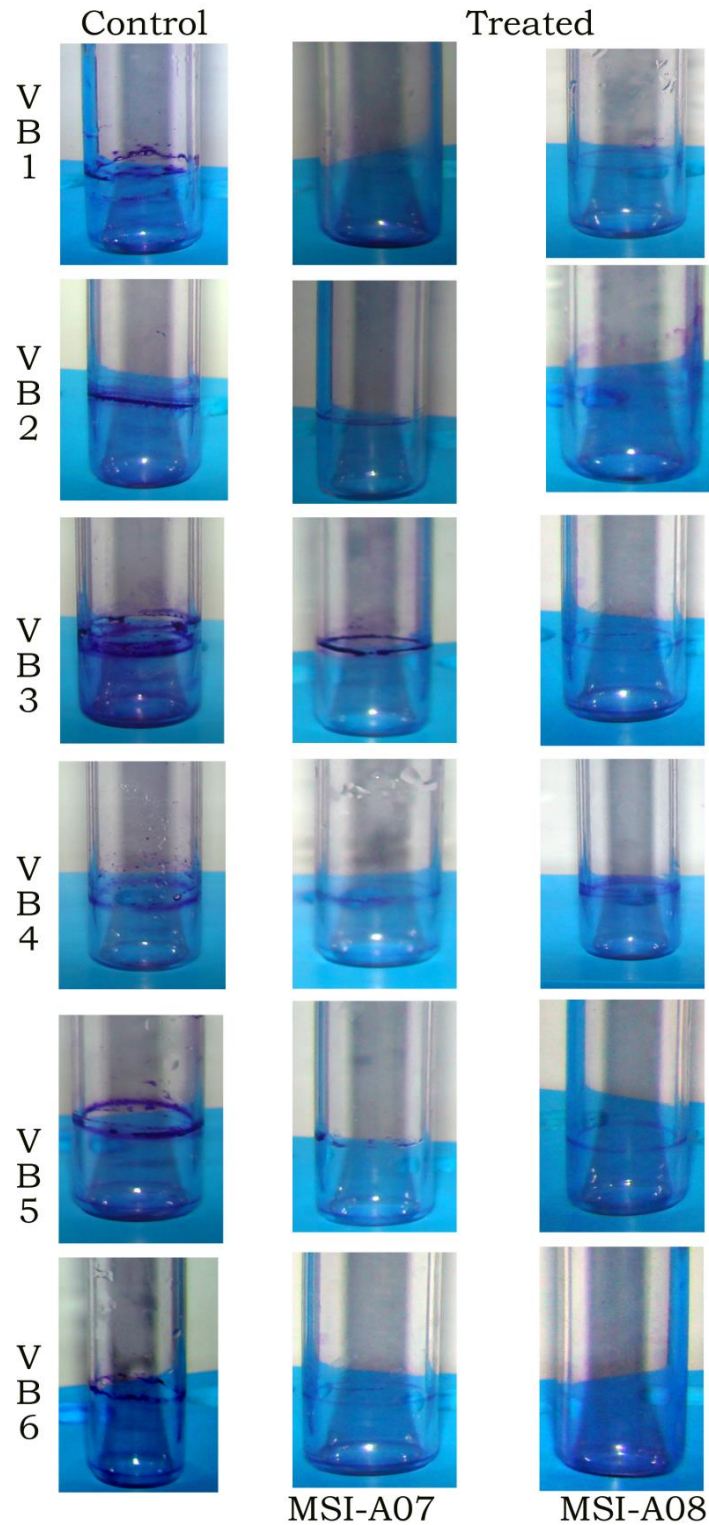


Plate 5.2. Direct observation demonstrating the antibiofilm potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

Kiran, G. et al., 2017. Development of antibiofilm biosurfactants from marine bacteria against shrimp *Vibrio* pathogens. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. . (Eds). Investigación y Desarrollo en Nutrición Acuícola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 284-302. ISBN 978-607-27-0822-8.

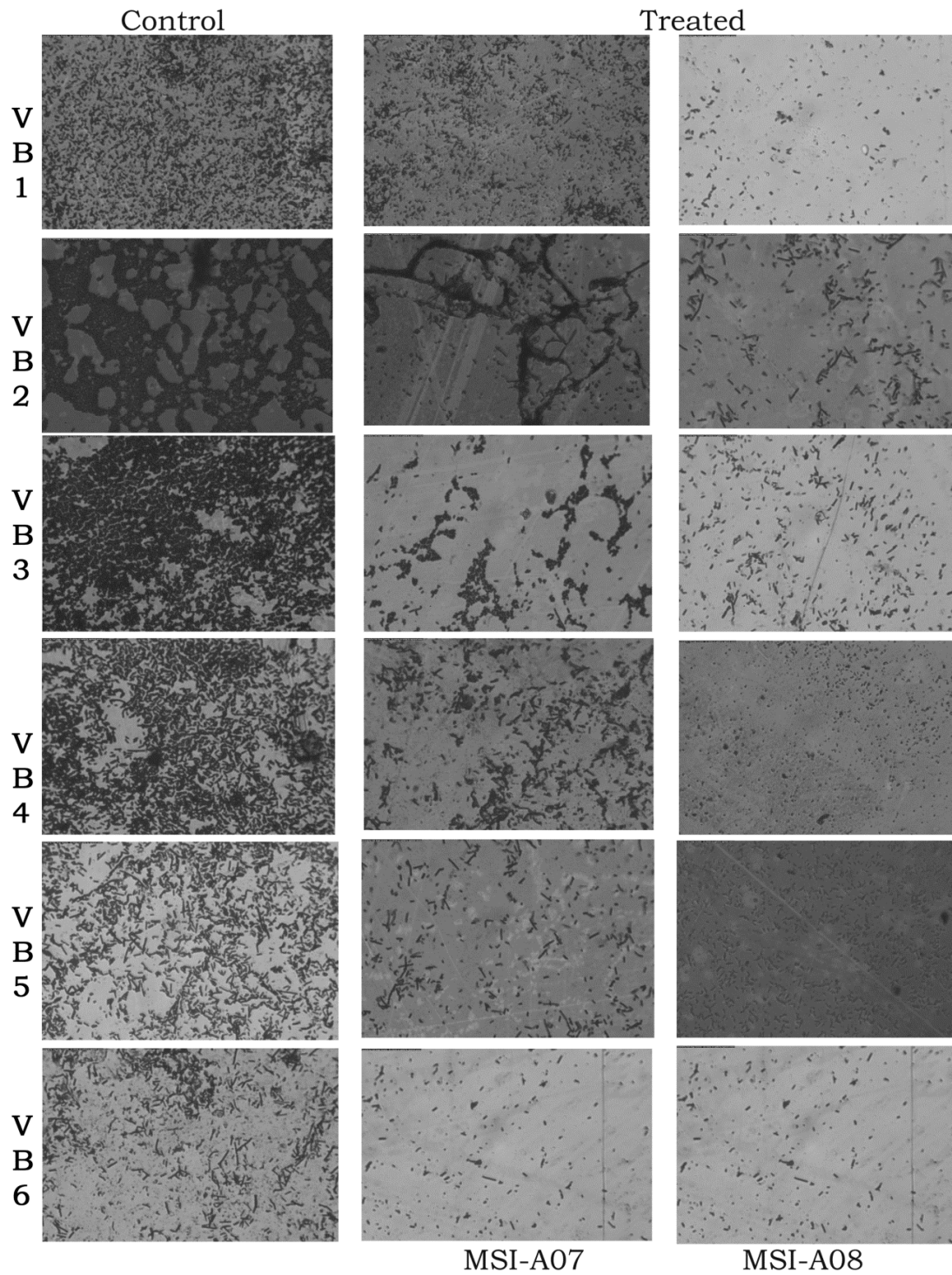


Plate 5.3. Phase contrast microscope images demonstrating the antibiofilm potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

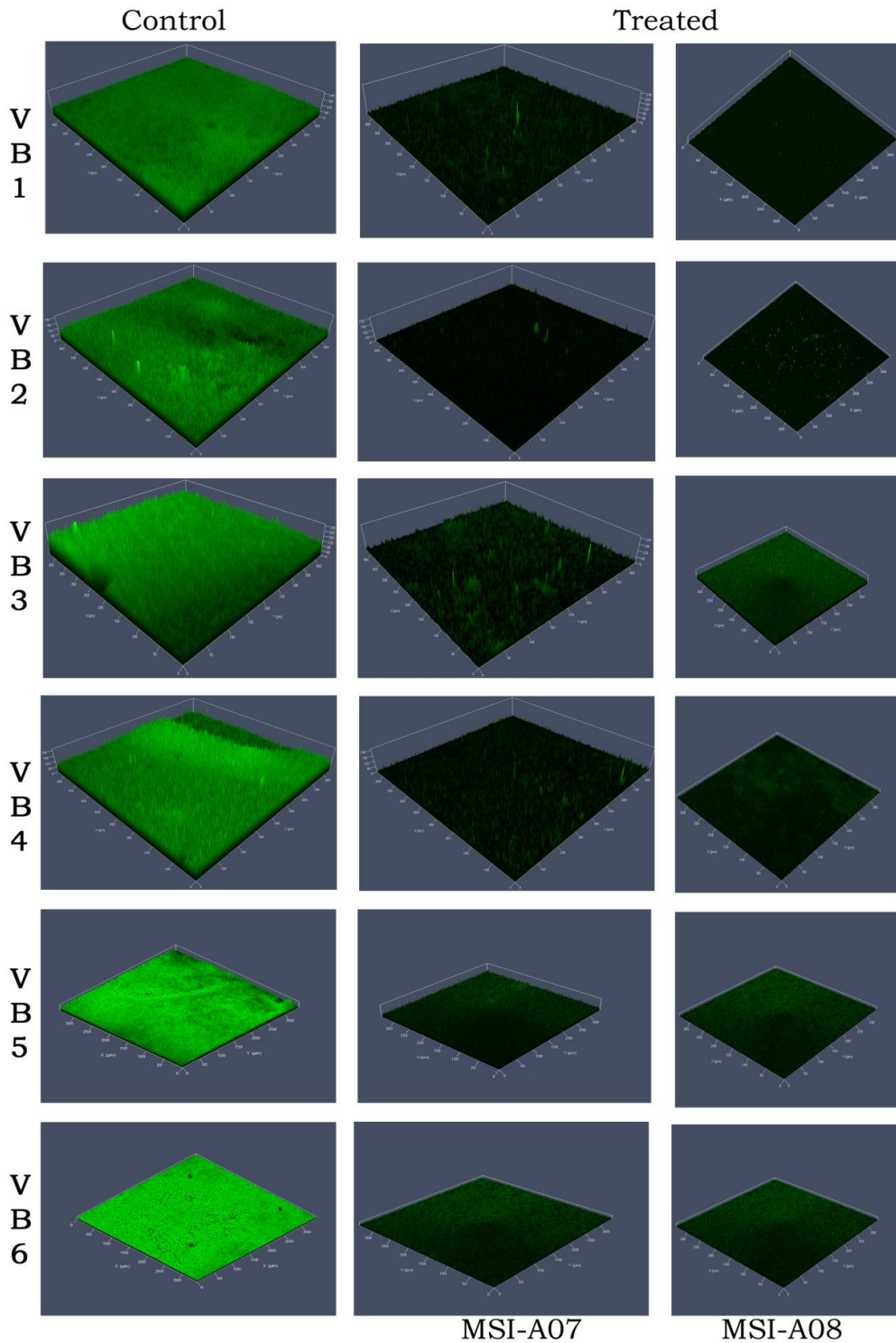


Plate 5.4. CLSM images demonstrating the antibiofilm potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

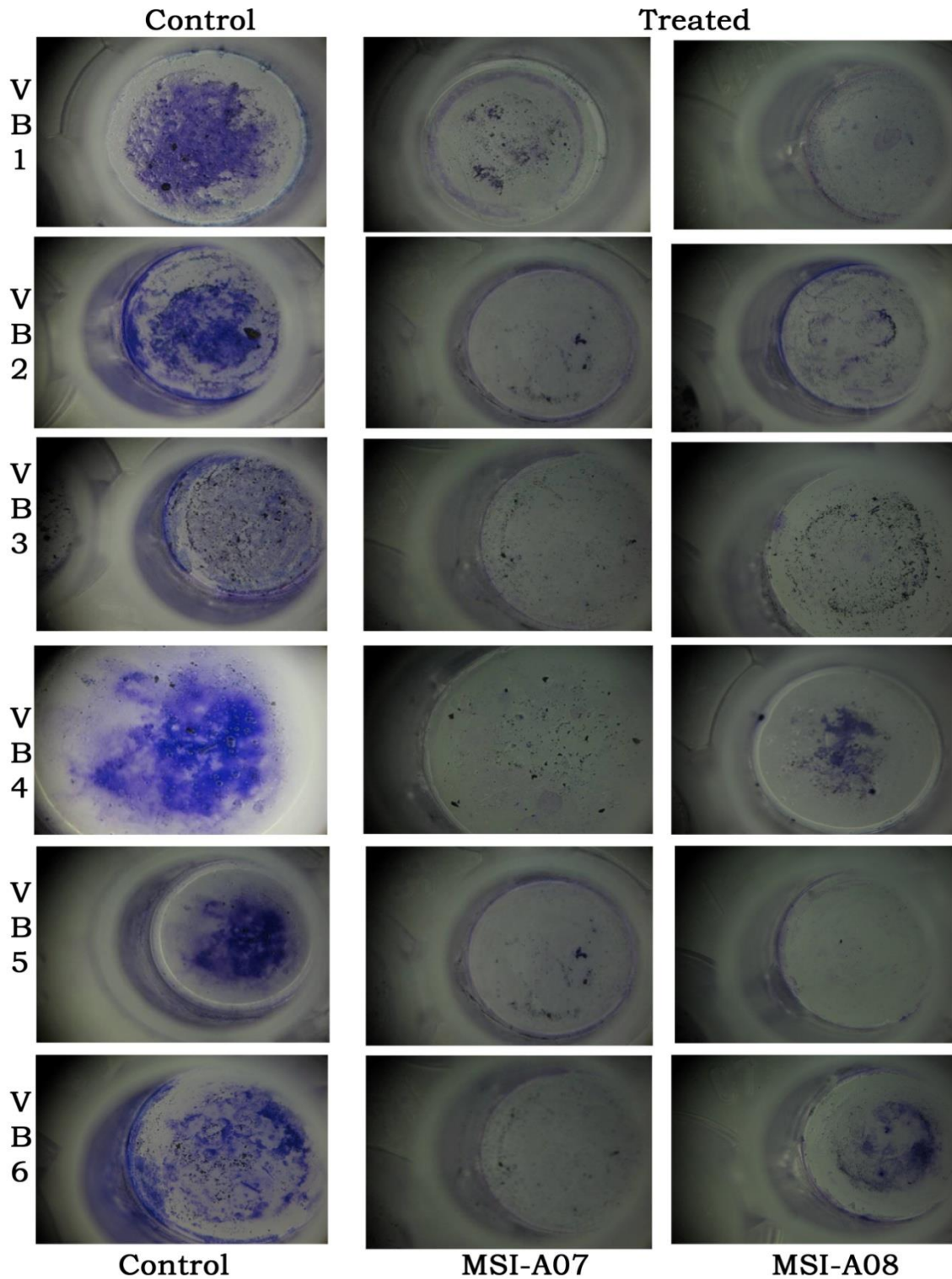


Plate 5.5. Stereozoom images demonstrating the antibiofilm potentials of MSI-A 07 and MSI-A 08 lipopeptide biosurfactants against shrimp pathogens

Kiran, G. et al., 2017. Development of antibiofilm biosurfactants from marine bacteria against shrimp *Vibrio* pathogens. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. . (Eds). Investigación y Desarrollo en Nutrición Acuícola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 284-302. ISBN 978-607-27-0822-8.

Microbial surfactants or biosurfactants are surface-dynamic amphipathic particles delivered by various microorganisms. As of late, microbial surfactants have been found to have a few properties of helpful and biomedical significance, e.g. antibacterial, antifungal and antiviral properties. The antimicrobial properties of the biosurfactants have been generally detailed. One valuable property of numerous biosurfactants that has not been examined widely is their antibiofilm action. Glycolipid delivered by *Brevibacterium casei* (Kiran *et al.* 2010) and rhamnolipid created by *Serratia marcescens* (Dusane *et al.* 2010) have indicated high antibiofilm. As of late Quinn *et al.* (2012) detailed the antibiofilm capability of lipopeptide separated from *Paenibacillus polymyxa*. The capacity of surfactants to repress biofilm development is depicted for the rhamnolipid surfactant of *P. aeruginosa PAO1* (Davey *et al.* 2003) and for lipopeptides created by the Gram-positive microscopic organisms *Lactobacillus*, *Bacillus* and *Streptococcus* (Busscher *et al.* 1997; Velraeds *et al.* 2000; Mireles *et al.* 2001). Be that as it may, the quantity of reports on advancement of novel antibiofilm biosurfactant is negligible. In spite of the fact that there have been few reports of novel antibiofilm biosurfactants, their biofilm interruption possibilities have not been investigated in points of interest. With this standpoint, the present examination was directed to assess biofilm interruption capability of lipopeptide biosurfactants extricated from marine actinobacteria *Nocardiopsis sp.* MSI-A 07 and *Streptomyces coeruleorubidus* MSI-A 08.

Bacterial development emerges rapidly not long after its connection to a strong substratum, which is the underlying stage in biofilm formation. For beginning couple of hours of development at first glance, the attachment is reversible (Marshall, 1994, Hoiby *et al.* 2001). In this manner, the anticipation of bacterial attachment at the extremely introductory stage can essentially lessen the danger of further biofilm formation. The both lipopeptide biosurfactants hindered the biofilm arrangement at its beginning time through lessening the microcolonies framed by shrimp pathogens. The both lipopeptide biosurfactants diminished biofilm formation up to 75 and 80% at a grouping of 30 µg/mL and 40 µg/mL against shrimp pathogens, separately. Comparative outcomes was accounted for by Rodrigues *et al.* (2006), he showed that rhamnolipids repress bacterial bond over a range changing from 21% to 81%. The 96 well microtiter plate measure is the most widely utilized examine for the identification of biofilm development (Christensen,

1985). Both inhibition of biofilm assay and microscopic observations obviously depicted that the both lipopeptide biosurfactants viably lessened and disrupted the microcolonies.

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A Meta-Analysis of Essential Amino Acid Requirements of Fish

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Abstract

There are wide variations in the published estimates of essential amino acid (EAA) requirements. Variations are thought to originate from different choices in mode of expression, response variable, and mathematical model. Here we conduct a meta-analysis of the growth-based, dose-response trials of 10 EAA in 22 teleost species: 249 studies were reviewed. Published data were entered in a spreadsheet and re-calculated across in a standard and systematic manner to allow comparisons. The considered unit of requirement were percentage of dry diet, g of EAA per MJ of digestible energy (DE), and g of ingested EAA per kg of metabolic body weight (MBW) per day. Response variables included growth in g per kg MBW per day and thermal-unit growth coefficient (TGC). Four mathematical models were also compared: broken-lime model (BLM), quadratic model (QM), broken-quadratic model (BQM), and saturation kinetic model (SKM). Results first indicate important differences in study quality, as 54% of the reviewed papers were excluded from the meta-analysis, often times because of poor growth or missing information. Additionally, the final dataset was greatly fragmented: 31% of the studied concerned rainbow trout, and lysine was the focus of 29% of all studies, leaving some species and EAA poorly covered. Comparisons of the requirement estimates show important variations between studies, even within species. With such variability there was no difference in requirement estimates calculated with different response variables. Similarly, this variability was not different between the three modes of expression, nor was it between mathematical models. However, there were significant effects of experimental design on the quality of fit of the models. Specifically, experiments that failed to produce a clear, plateauing dose-response curve had greatly increased probability to yielding absurd results (e.g. negative requirement). Finally, the present study emphasizes the critical need of a global, standard and systematic system to report and capture results from nutrition trials.

Keywords: Meta-analysis, amino acid, requirement

1 Introduction

Aquafeed formulations are constantly refined, updated, and adjusted to reflect progress in nutrition science, as well as the variability in ingredients price and availability. Feeds must remain cost-effective and deliver nutrients efficiently to the animal in order to promote growth and health while minimizing nutrient discharge in the effluent. As a result, fish feed are increasingly formulated at the nutrient level, as is commonly done in other livestock, e.g., poultry. Naturally, the first prerequisite to this goal is a precise knowledge of essential amino acid (EAA) requirement.

However, a number of reviews have identified significant gaps in our understanding of EAA requirements of teleosts and highlighted the great variability in estimates of EAA requirements amongst and within species (Bureau and Encarnaç o, 2006; Cowey, 1994; Lall and Anderson, 2005; NRC, 1993, 2011; Wilson, 1989). For example, in rainbow trout *Oncorhynchus mykiss* lysine requirement range from 1.3 to 2.9% of the diet (3.7-7.3% of crude protein, CP), and arginine requirement estimates span from 1.4 to 2.2% of the diet. Genetic variations likely account for but a fraction of such different estimates. Rather, reasons explaining these discrepancies can be found in different methodological approaches. Calculation of a requirement estimate requires choosing a mode of expression, a response variable, and a mathematical model to describe their relationship. Modes of expression include percentage of the diet or crude protein, or in g/MJ digestible energy. The choice of mode of expression is a matter of much debate, and reflects the various assumptions authors make when considering what affects the requirements or not (Bureau and Encarnaç o, 2006; Bureau, 2008). Because these assumptions contradict each other, the same diet can be deemed deficient or not depending on the mode of expression of EAA requirement. Similarly, the choice of the response variable likely influences the estimates of requirements, as it introduces another level of variability between experiments. Responses commonly used in this context include expressions of weight gain (absolute weight gain, specific growth rate, percentage increase, thermal-unit growth coefficient, etc.), feed efficiency, nitrogen retention, or, more rarely, blood EAA concentration. Most studies consider growth as the primary response, but they do not all use the same starting weight in a given species. This is also problematic, since biological processes governing growth in fish are quite dynamic and

change significantly during the production cycle (Dumas *et al.* 2007), and EAA requirements likely change as well. Mathematical models complete the triptych of the analytical aspect of nutrient requirement estimation. Different models have been used to describe the fish biological response, which typically follows the law of “diminishing return”. The broken-line model (BLM) has been heavily used for the past two decades. However, when compared to other non-linear models, the BLM consistently returns lower estimates (Hernandez-Llamas, 2009). In contrast, Shearer (2000) noted that non-linear models could yield requirement estimates up to five times the published requirement.

Clearly, there is a dire need to re-visit fish EAA requirements. Thus, the present study first extensively reviewed the existing literature on EAA requirements in fish and systematically standardized the relevant data. The resulting dataset was then analyzed using various modes of expression, responses and mathematical models to re-evaluate the EAA requirement in these commercially important fish species.

2 Methods and calculations

2.1 Search of the literature and selection criteria

An extensive search of the EAA requirement literature was conducted using online databases, peer-reviewed journals, and books. At this point, the sole selection criterion was to only keep studies on commercially-relevant species (e.g., salmonidae, cyhlidae, cyprinidae), resulting in 286 articles. There was among these a great variety of objectives, experimental designs, and analytical methodologies employed. Therefore, additional selection criteria were applied to the dataset to eliminate studies unsuitable to the present meta-analysis.

Since growth was by far the most studied type of response, only studies based on growth trials in response to graded dietary levels of a unique AA were kept. Thus experiments based on the measurement of plasma AA levels, or on the ideal protein theory, were not included (37 studies out of the initial 286).

Other selection criteria were as follow: husbandry practices must be adequate for the species of interest, including water quality. Information on temperature and experiment duration must be provided. All experimental diets must be adequately characterized and meet all other

known nutritional requirements. Studies must use five or more experimental diets in order to ensure the accuracy of the non-linear regressions analyses. Data, especially diet composition, must be reported on a dry-matter basis, or enough information must be provided to compute dry-matter composition of the diets. Studies where mortality was high and uncorrelated with EAA graded levels were not included. Finally, initial and final individual body weight, as well as feed intake, must be reported, or enough information must be provided to calculate them.

2.2 Variables and models combinations

The meta-analysis was conducted as a 3x2x4 array: three factor variables (percentage of EAA of interest in the dry diet, amount of EAA per MJ of digestible energy, and the amount of the EAA of interest ingested during the experiment), and two response variables (weight gain per kg of metabolic body weight and thermal-unit growth coefficient, TGC). Together, they form six pairs of variable. Finally, four regression models (broken-line, quadratic, broken-quadratic, and saturation-kinetic models) were used to estimate the EAA requirements for each of the variable pair and for each selected study.

2.2.1 Calculated factor and response variables

Weight gain, on a metabolic body weight-basis (WG_{MBW} , in g/kgMBW/d)

$$WG_{MBW} = \frac{FBW - IBW}{\left[\left(\frac{FBW^{1/3} + IBW^{1/3}}{2} \right)^3 \times 10^{-3} \right]^{0.8}} \times d$$

Thermal-unit growth coefficient (TGC, dimensionless)

$$TGC = 100 \times \frac{FBW^{1/3} - IBW^{1/3}}{t \times d}$$

AA dietary level to digestible energy (AA_{DE} , in g/MJ_{DE})

$$AA_{DE} = \frac{AA_D \times 10}{GE_D \times ADC_{GE}}$$

AA ingested (AA_I , in g)

$$AA_I = \frac{AA_D}{100} \times FI$$

Where IBW and FBW are the individual initial and final body weight (in g) respectively; d is the duration of the experiment (in days), t is the average temperature during the experiment (in °C); AA_D is the diet content of the amino acid of interest (% dry matter); GE_D is the gross energy content of the diet (in MJ.kg⁻¹); ADC_{GE} is the apparent digestibility coefficient of gross energy (%); FI is the feed intake during the experiment (in g, dry matter basis).

2.2.2 Models

The broken line model (BLM) is defined as follow:

$$Y = a_1X + b_1 + (a_2 - a_1)\delta X + (b_2 - b_1)\delta + \varepsilon$$

$$\delta=0 \text{ if } X \leq X_{bp}, \text{ or } \delta=1 \text{ if } X \geq X_{bp}$$

Where Y and X are the response and independent variables, respectively; X_{bp} is the abscissa of the breaking point thus defining the AA requirement level; b_1 and b_2 are the intercepts, and a_1 and a_2 the slopes, of the 2 lines, respectively. Note that a_2 was not constrained to zero. The quadratic model (QM) is defined as follow:

$$Y = aX^2 + bX + c + \varepsilon$$

Where Y and X are the response and independent variables, respectively, while a , b , and c are equation parameters. The amino acid requirement is the abscissa of the smaller root that solve the parabola's equation for $Y=95\%$ of the maximum response.

The following equation defines the broken-quadratic model (BQM):

$$Y = aX^2 + bX + c + a(X_{jp}^2 - X^2)\delta + b(X_{jp} - X)\delta + \varepsilon$$

$$\delta=0 \text{ if } X \leq X_{jp}, \text{ or } \delta=1 \text{ if } X \geq X_{jp}$$

Where Y and X are the response and independent variables, respectively, and a , b , and c are equation parameters. X_{jp} is the abscissa of the junction point thus defining the AA requirement level. Note that since X_{jp} is a constant, the curve becomes a line of slope zero for amino acid levels above the estimated requirement.

Finally, the saturation-kinetic model (SKM) is defined as follow:

$$Y = \frac{b \times c + aX^d}{c + X^d} + \varepsilon$$

Where Y and X are the response and independent variables, respectively; a is the asymptote to the maximum response; b is the intercept; c is a coefficient; d is a kinetic order. The amino acid requirement is defined as the abscissa where the response variable reaches 95% of its predicted maximum (i.e. 95% of a).

Estimations of EAA requirements were computed for each of the independent/dependent variable couples and for each of the 4 models. Outputs were classified into the following categories: “no fit” indicates that the model failed to converge, and therefore no estimate of requirement was generated. “Within” means that the model converged and the estimated requirement fell within the range of experimental EAA levels tested in a given study. “Outside” signifies that the model successfully converged, but the estimate requirement fell outside of the EAA range of the experimental diets. “No break”, for the BLM and BQM, indicates that only one of the two curves calculated by the model fitted, hence no breaking point could be determined. Finally, the quadratic-based models (BQM and QM) sometimes converged to a positive a , thus causing the model to have a minimum instead of a maximum. This behaviour was noted as “minimum”.

2.3 Statistical analysis

Models were programmed and run using SAS 9.4 (SAS Institute). Qualities of fit and coefficient of variation of requirement estimates were tested by logistic tests and a two-way ANOVA (variable pairs, models), respectively.

3 Results and discussion

3.1 Screening results

A total of 348 peer-reviewed articles spanning 52 years (1964 to 2016) were found during the literature search, out of which 48 were not growth-based trials – e.g. based on ideal-protein formulations, or on changes in plasma AA levels, review articles – hence could not be included in the meta-analysis. However, other selection criteria rejected over half (52%) of the 300 remaining studies from the meta-analysis (Figure 1). The main reasons for rejection included poor growth, missing information (e.g. dry matter data, feed intake), or too few graded levels (

Table 1). The final dataset (Table 2) 145 studies which covered 37 species of fish, and 11 EAA. About 72% of the studies used 6 or 7 graded levels of the EAA of interest. One study used 24 graded levels. The variables most commonly used for the estimation of the requirement are the EAA content of the diet (% dry matter) and weight gain (as SGR or % of IBW) as the independent and response variable, respectively. The most commonly used models are the BLM and QM.

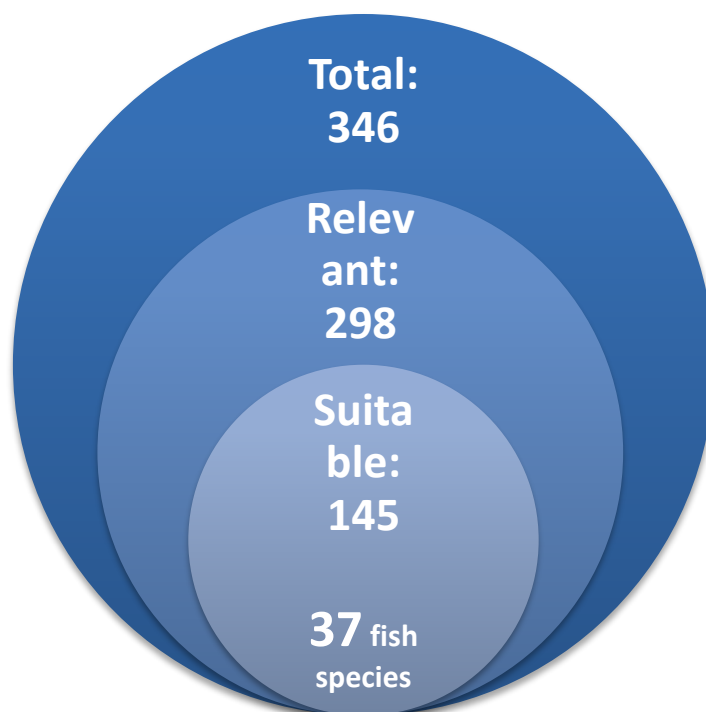


Figure 1: Diagram illustrating the screening of studies, from the total number found to the number of suitable studies that constituted our working data set

Table 1: Study rejection

Reason for rejection	% of rejected studies
Restricted feeding	3%
Poor growth	34%
Too few dietary levels	10%
Lack of intake data	44%
Lack of dry matter data	17%
Lack of raw growth data	2%

The number of excluded studies is striking. It highlights the uneven quality of the research effort in this area. It also exacerbated the fragmentation of the final dataset, both in terms of species and EAA. For instance, 25% of the studies focused on rainbow trout, whereas studies on other species such as European seabass, Japanese flounder, or Nile tilapia each represent 3% or less of the dataset. Similarly, lysine is by far the most-studied EAA (27% of the studies), while only five studies (3% of the dataset, Figure 2) describe the requirement of

phenylalanine. Moreover, these five studies were performed on five different species. Such fragmentation constitutes a major challenge for the understanding and definition of EAA requirements, *let al* one their rationalization.

3.2 Estimates of EAA requirements

There were least two studies in 13 of the possible combinations of species and EAA (Table 3). These 13 combinations reflect the most-studied EAA (arginine, isoleucine, lysine, methionine, threonine, and tryptophan) and species (channel catfish, hybrid striped bass, rainbow trout, and Chinook, Coho and Atlantic salmon). Estimations are generally well in agreement with those reported by the NRC (2011): e.g., in % of diet dry matter, 1.66% arginine in rainbow trout, 1.53% lysine in channel catfish, or 0.61% methionine in hybrid striped bass.

Table 2: Summary of studies included in the final dataset

Studied EAA	Species	# of diet	EAA range in diets (% DM)	Av. water temp.	Av. IBW (g)	$\frac{maxFBW}{IBW}$	$\frac{minFBW}{maxFBW}$	Exp. Duration (days)	Reference
Arg	<i>Clarias hybrid</i>	6	1.06-2.28	26.0	6.71	6.23	0.49	28	(Singh and Khan, 2007)
	<i>Ictalurus punctatus</i>	8	0.20-1.60	26.7	200	2.21	0.51	56	(Robinson <i>et al.</i> 1981)
	<i>Heteropneustes fossilis</i>	6	0.85-2.13	23.5	4.83	3.77	0.49	56	(Ahmed, 2013)
	<i>Labeo rohita</i>	6	0.47-1.77	28.0	0.62	5.00	0.37	56	(Abidi and Khan, 2009)
	<i>Cirrhinus mrigala</i>	6	1.00-2.25	26.5	0.61	4.21	0.55	56	(Ahmed and Khan, 2004b)
	<i>Ctenopharyngodon idella</i>	6	0.93-2.41	27.8	3.85	5.53	0.50	70	(Gao <i>et al.</i> 2015)
	<i>Catla catla</i>	6	0.98-2.23	27.9	0.60	12.41	0.46	84	(Zehra and Khan, 2013a)
	<i>Micropterus salmoides</i>	6	1.70-3.01	28.0	24.93	2.77	0.88	56	(Zhou <i>et al.</i> 2012)
	<i>Oncorhynchus kisutch</i>	6	1.20-3.60	10.0	2.90	3.30	0.65	70	(Klein and Halver, 1970)
	<i>Oncorhynchus tshawytscha</i>	6	1.60-3.60	10.0	3.48	2.89	0.79	70	(Klein and Halver, 1970)
	<i>Oncorhynchus kisutch</i>	6	0.00-5.27	15.0	0.91	5.90	0.87	56	(Luzzana <i>et al.</i> 1998)
	<i>Oncorhynchus mykiss</i>	12	0.5-2.38	17.0	50.67	3.54	0.54	51	(Rodehutschord <i>et al.</i> 1995a)
	<i>Oncorhynchus mykiss</i>	8	0.47-2.50	15.0	12.60	3.35	0.64	42	(Kim <i>et al.</i> 1992b)
	<i>Oncorhynchus mykiss</i>	6	0.69-3.83	15.0	2.50	14.08	0.65	84	(Cho <i>et al.</i> 1992)
	<i>Oncorhynchus mykiss</i>	7	0.69-3.00	15.0	1.30	19.46	0.72	84	(Cho <i>et al.</i> 1992)
	<i>Oncorhynchus mykiss</i>	5	0.80-2.80	10.0	7.30	4.08	0.60	84	(Walton <i>et al.</i> 1986)
	<i>Oncorhynchus mykiss</i>	5	1.72-3.61	17.0	9.30	9.46	0.74	84	(Fournier <i>et al.</i> 2003)
	<i>Salmo salar</i>	5	1.13-2.86	6.5	385	1.50	0.91	126	(Berge <i>et al.</i> 1997)
	<i>Salmo salar</i>	5	1.10-3.20	15.0	111.9	5.00	0.87	98	(Lall <i>et al.</i> 1994)
	<i>Psetta maxima</i>	5	1.79-4.19	17.0	7.40	8.03	0.43	84	(Fournier <i>et al.</i> 2003)

	<i>Sparus macrocephalus</i>	6	1.85-3.46	28.0	10.51	4.87	0.84	56	(Zhou et al. 2010b)
	<i>Lates calcarifer</i>	6	0.73-3.73	29.0	2.56	7.96	0.64	84	(Murillo-Gurrea et al. 2001)
	<i>Rachycentron canadum</i>	6	1.76-3.75	30.5	3.38	13.11	0.76	63	(Ren et al. 2014)
	<i>Paralichthys olivaceus</i>	6	1.25-3.25	20.6	1.85	4.25	0.53	40	(Alam et al. 2002)
His	<i>Ictalurus punctatus</i>	9	0.10-0.80	26.7	200	3.31	0.39	56	(Wilson et al. 1980)
	<i>Cirrhinus mrigala</i>	6	0.25-1.50	27.5	0.61	3.95	0.60	56	(Ahmed and Khan, 2005b)
	<i>Catla catla</i>	6	0.25-0.96	28.1	0.65	14.33	0.03	84	(Zehra and Khan, 2016c)
	<i>Heteropneustes fossilis</i>	6	0.90-1.95	27.9	5.27	14.33	0.08	84	(Khan and Abidi, 2014)
	<i>Oncorhynchus mykiss</i>	12	0.26-1.35	17.0	40.25	4.22	0.71	53	(Rodehutscord et al. 1997)
	<i>Oncorhynchus keta</i>	7	0.13-1.92	14.8	1.58	2.46	0.64	28	(Akiyama et al. 1985)
	<i>Oncorhynchus kisutch</i>	5	0.50-1.30	10.0	3.17	3.00	0.88	70	(Klein and Halver, 1970)
Ile	<i>Ictalurus punctatus</i>	7	0.30-1.30	26.7	200	2.95	0.47	56	(Wilson et al. 1980)
	<i>Labeo rohita</i>	6	0.75-1.99	26.7	0.62	4.46	0.62	56	(Khan and Abidi, 2007b)
	<i>Cirrhinus mrigala</i>	6	0.50-1.75	27.7	0.61	4.18	0.52	56	(Ahmed and Khan, 2006)
	<i>Catla catla</i>	6	0.51-1.73	27.8	0.61	11.69	0.43	84	(Zehra and Khan, 2013c)
	<i>Oncorhynchus mykiss</i>	12	0.50-1.53	16.0	45.65	3.43	0.55	59	(Rodehutscord et al. 1997)
	<i>Salvelinus namaycush</i>	7	0.54-1.26	8.3	3.20	4.00	0.94	84	(Hughes et al. 1983)
	<i>Oncorhynchus tshawytscha</i>	6	0.50-1.50	10.0	0.82	4.26	0.68	70	(Chance et al. 1964)
	<i>Oncorhynchus tshawytscha</i>	6	0.50-1.50	10.0	0.79	4.14	0.61	70	(Chance et al. 1964)
Leu	<i>Ictalurus punctatus</i>	8	0.60-2.00	26.7	200	2.87	0.76	56	(Wilson et al. 1980)

	<i>Labeo rohita</i>	6	0.74-1.99	26.7	0.59	4.00	0.46	56	(Abidi and Khan, 2007)
	<i>Cirrhinus mrigala</i>	6	0.75-2.00	27.7	0.64	4.08	0.55	56	(Ahmed and Khan, 2006)
	<i>Oreochromis niloticus</i>	6	0.53-1.81	29.0	1.94	10.77	0.70	56	(Gan <i>et al.</i> 2016)
	<i>Salvelinus namaycush</i>	7	0.96-2.24	8.3	3.20	4.03	0.84	84	(Hughes <i>et al.</i> 1983)
	<i>Oncorhynchus tshawytscha</i>	6	1.00-3.10	10.0	2.49	2.11	0.60	56	(Chance <i>et al.</i> 1964)
	<i>Oncorhynchus mykiss</i>	12	1.00-4.20	15.7	49.33	3.77	0.66	53	(Rodehutsord <i>et al.</i> 1997)
	<i>Lateolabrax japonicus</i>	6	0.90-3.88	29.0	8.00	4.90	0.85	56	(Li <i>et al.</i> 2015)
Lys	<i>Hybrid striped bass</i>	6	1.14-2.37	24.5	7.0	6.76	0.78	63	(Keembiyehetty and Gatlin, 1992)
	<i>Hybrid striped bass</i>	8	1.20-2.60	28.0	5.5	10.46	0.85	77	(Griffin <i>et al.</i> 1992)
	<i>Ictalurus punctatus</i>	6	0.75-2.00	26.7	202	2.50	0.66	44	(Wilson <i>et al.</i> 1977)
	<i>Ictalurus punctatus</i>	6	0.70-2.00	26.7	201	2.69	0.51	56	(Robinson <i>et al.</i> 1980b)
	<i>Pelteobagrus fulvidraco</i>	6	1.73-4.19	28.0	1.50	8.00	0.70	56	(Cao <i>et al.</i> 2012)
	<i>Lepomis macrochirus</i>	7	1.19-3.29	22.3	26.93	1.50	0.83	56	(Masagounder <i>et al.</i> 2011)
	<i>Ctenopharyngodon idella</i>	6	0.71-3.16	28.7	3.15	4.49	0.64	90	(Wang <i>et al.</i> 2005)
	<i>Cirrhinus mrigala</i>	6	1.48-2.78	26.5	0.66	3.95	0.59	40	(Ahmed and Khan, 2004a)
	<i>Catla catla</i>	6	1.22-2.47	26.8	0.59	12.32	0.45	84	(Zehra and Khan, 2013b)
	<i>Piaractus mesopotamicus</i>	6	0.82-2.10	29.6	6.67	7.08	0.61	90	(Abimorad <i>et al.</i> 2010)
	<i>Oncorhynchus keta</i>	8	0.38-4.00	14.8	1.27	3.66	0.57	28	(Akiyama <i>et al.</i> 1985)

<i>Oncorhynchus mykiss</i>	24	0.45-5.80	15.7	50.9	3.65	0.32	55	(Rodehutsord <i>et al.</i> 1997)
<i>Oncorhynchus mykiss</i>	6	1.60-2.42	14.5	14.9	6.00	0.83	56	(Cheng <i>et al.</i> , 2003)
<i>Oncorhynchus mykiss</i>	8	1.37-3.66	15.0	5.0	16.38	0.65	84	(Wang <i>et al.</i> unpublished)
<i>Oncorhynchus mykiss</i>	6	1.27-2.32	15.0	24.0	7.20	0.69	84	(Encarnacao <i>et al.</i> 2004)
<i>Oncorhynchus mykiss</i>	6	1.33-2.49	15.0	24.0	7.54	0.75	84	(Encarnacao <i>et al.</i> 2004)
<i>Oncorhynchus mykiss</i>	8	0.72-1.60	15.0	13.7	3.52	0.65	42	(Kim <i>et al.</i> 1992b)
<i>Oncorhynchus mykiss</i>	8	0.21-3.01	11.5	17.0	4.61	0.40	71	(Pfeffer <i>et al.</i> 1992)
<i>Oncorhynchus mykiss</i>	6	1.03-2.02	15.0	0.92	5.15	0.57	42	(Nang Thu <i>et al.</i> 2007)
<i>Oncorhynchus mykiss</i>	6	1.00-2.03	15.0	0.92	3.64	0.58	42	(Nang Thu <i>et al.</i> 2007)
<i>Oncorhynchus mykiss</i>	6	1.06-2.05	15.0	0.92	5.18	0.54	42	(Nang Thu <i>et al.</i> 2007)
<i>Oncorhynchus mykiss</i>	6	1.03-2.02	15.0	0.92	5.32	0.49	30	(Nang Thu <i>et al.</i> 2009)
<i>Oncorhynchus mykiss</i>	6	0.63-1.43	15.0	0.92	3.79	0.48	30	(Nang Thu <i>et al.</i> 2009)
<i>Oncorhynchus mykiss</i>	6	1.03-2.02	15.0	0.92	4.51	0.47	30	(Nang Thu <i>et al.</i> 2009)
<i>Oncorhynchus mykiss</i>	7	1.04-2.60	15.0	5.19	10.87	0.50	84	(Walton <i>et al.</i> 1984b)
<i>Salmo salar</i>	7	1.24-2.94	15.0	4.72	2.71	0.61	70	(Anderson <i>et al.</i> 1993)
<i>Salmo salar</i>	12	1.20-2.38	15.2	42.12	2.63	0.64	76	(Hauler <i>et al.</i> 2007)
<i>Salmo salar</i>	7	1.26-4.15	8.0	643	1.60	0.89	85	(Espe <i>et al.</i> 2007)
<i>Lates calcarifer</i>	6	1.00-2.50	27.0	13.12	4.58	0.70	84	(Murillo-Gurrea <i>et al.</i> 2001)
<i>Sparus macrocephalus</i>	6	2.08-4.05	18.0	9.12	4.14	0.85	56	(Zhou <i>et al.</i> 2009, 2010a)
<i>Pagrus major</i>	6	1.19-3.44	22.5	1.7	8.06	0.58	56	(Forster and Ogata, 1998)
<i>Paralichthys olivaceus</i>	6	1.04-3.16	19.5	3.0	5.33	0.45	63	(Forster and Ogata, 1998)

	<i>Psetta maxima</i>	7	1.20-3.11	18.0	18.1	1.82	0.88	56	(Peres and Oliva-Teles, 2008)
	<i>Rachycentron canadum</i>	6	1.15-3.25	28.5	1.26	19.09	0.66	56	(Zhou et al. 2007)
	<i>Sciaenops ocellatus</i>	7	1.00-2.50	27.0	6.72	4.57	0.68	56	(Craig and Gatlin, 1992)
	<i>Dicentrarchus labrax</i>	5	1.33-2.72	25.5	0.85	7.73	0.65	70	(Tibaldi and Lanari, 1991)
Met	<i>Hybrid striped bass</i>	6	0.38-1.60	24.0	4.8	5.12	0.20	56	(Keembiyehetty and Gatlin, 1993)
	<i>Hybrid striped bass</i>	5	0.60-1.00	24.0	8.6	5.38	0.51	56	(Keembiyehetty and Gatlin, 1993)
	<i>Oreochromis niloticus</i>	5	0.56-0.80	26.2	1.31	6.05	0.78	43	(Furuya et al. 2008)
	<i>Megalobrama amblycephala</i>	6	0.39-1.54	28.5	3.34	7.33	0.73	63	(Liao et al. 2014)
	<i>Catla catla</i>	6	0.56-1.81	26.9	0.65	14.77	0.39	84	(Zehra and Khan, 2016b)
	<i>Ictalurus punctatus</i>	7	0.25-1.75	26.7	202	2.39	0.71	44	(Harding et al. 1977)
	<i>Ictalurus punctatus</i>	7	0.35-1.55	24.8	14.36	2.34	0.85	42	(Cai and Burtle, 1996)
	<i>Myxocyprinus asiaticus</i>	6	0.64-1.89	27.9	1.72	6.10	0.67	56	(Chu et al. 2014)
	<i>Pseudobagrus ussuriensis</i>	6	0.49-2.08	24.0	0.60	6.16	0.72	56	(Wang et al. 2016)
	<i>Oncorhynchus mykiss</i>	12	0.22-1.09	16.8	43.04	3.25	0.43	49	(Rodehutscord et al. 1995b)
	<i>Oncorhynchus mykiss</i>	12	0.21-1.10	16.8	43.46	3.59	0.42	49	(Rodehutscord et al. 1995b)
	<i>Oncorhynchus mykiss</i>	7	0.43-2.07	15.0	3.13	15.48	0.80	112	(Cowey et al. 1992)
	<i>Oncorhynchus mykiss</i>	6	0.23-0.80	15.0	15.4	3.08	0.67	42	(Kim et al. 1992a)
	<i>Salvelinus alpinus</i>	6	0.43-0.91	12.0	20.55	5.04	0.34	112	(Simmons et al. 1999)
	<i>Salmo salar</i>	6	0.69-1.29	8.5	495	2.44	0.87	85	(Espe et al., 2008)

	<i>Paralichthys olivaceus</i>	6	0.53-2.03	21.6	2.8	4.45	0.34	40	(Alam et al. 2000)
	<i>Sparus macrocephalus</i>	6	0.75-2.35	28.0	14.22	4.36	0.84	56	(Zhou et al. 2011)
	<i>Sciaenops ocellatus</i>	7	0.35-1.85	27.0	0.9	14.39	0.19	56	(Moon and Gatlin III, 1991)
	<i>Trachinotus ovatus</i>	6	0.86-1.45	29.5	12.40	6.58	0.64	56	(Niu et al. 2013)
	<i>Rachycentron canadum</i>	6	0.61-1.68	29.0	11.62	7.37	0.62	56	(Zhou et al. 2006)
	<i>Lates calcarifer</i>	6	0.62-1.26	26.5	2.61	11.10	0.86	84	(Coloso et al. 1999)
Phe	<i>Ictalurus punctatus</i>	7	0.20-0.80	26.7	199	2.50	0.60	56	(Robinson et al. 1980a)
	<i>Cirrhinus mrigala</i>	6	0.50-1.75	26.2	0.56	3.88	0.54	56	(Ahmed, 2009)
	<i>Labeo rohita</i>	6	0.39-1.64	26.0	0.58	4.14	0.60	56	(Khan and Abidi, 2007a)
	<i>Catla catla</i>	6	0.39-1.62	27.2	0.68	13.10	0.43	84	(Zehra and Khan, 2014)
	<i>Oncorhynchus mykiss</i>	7	0.26-1.75	15.0	12.7	2.91	0.55	42	(Kim, 1993)
Thr	<i>Ictalurus punctatus</i>	7	0.30-1.20	26.7	200	2.79	0.61	56	(Wilson et al. 1978)
	<i>Hybrid striped bass</i>	6	0.49-1.75	26.5	9.8	4.11	0.73	49	(Keembiyehetty and Gatlin, 1997)
	<i>Hybrid striped bass</i>	6	0.49-1.25	26.5	3.0	6.46	0.69	56	(Keembiyehetty and Gatlin, 1997)
	<i>Cirrhinus mrigala</i>	6	1.00-2.25	26.7	0.52	4.05	0.56	56	(Ahmed et al. 2004)
	<i>Labeo rohita</i>	6	0.71-2.15	27.0	0.58	4.14	0.49	56	(Abidi and Khan, 2008)
	<i>Catla catla</i>	6	0.74-1.93	26.9	0.60	14.32	0.40	84	(Zehra and Khan, 2016a)
	<i>Oreochromis niloticus</i>	6	0.71-2.46	28.8	6.00	3.49	0.63	56	(He et al. 2016)
	<i>Oreochromis niloticus</i>	5	0.89-1.54	28.5	563.30	1.47	0.92	28	(Michelato et al. 2016)
	<i>Megalobrama amblycephala</i>	5	0.61-2.49	27.0	3.01	4.30	0.70	63	(Habte-Tsion et al. 2015)
	<i>Oncorhynchus keta</i>	8	0.24-2.80	14.8	1.19	2.55	0.53	28	(Akiyama et al. 1985)
	<i>Oncorhynchus mykiss</i>	11	0.50-2.18	14.7	1.84	3.52	0.50	24	(Bodin et al. 2008)

	<i>Oncorhynchus mykiss</i>	11	0.50-2.10	17.0	50.74	3.25	0.61	51	(Rodehutscord <i>et al.</i> 1995a)
	<i>Salmo salar</i>	7	0.00-0.75	14.5	0.80	2.90	0.46	36	(Rollin <i>et al.</i> 2006)
	<i>Salmo salar</i>	11	0.50-2.18	14.7	0.80	3.00	0.47	36	(Bodin <i>et al.</i> 2008)
	<i>Dicentrarchus labrax</i>	6	0.81-2.63	21.1	7.50	2.80	0.89	65	(Tibaldi and Tulli, 1999)
	<i>Sciaenops ocellatus</i>	6	0.49-1.25	27.0	2.80	8.09	0.41	56	(Boren and Gatlin III, 1995)
Trp	<i>Ictalurus punctatus</i>	6	0.05-0.40	26.7	200	2.87	0.47	56	(Wilson <i>et al.</i> 1978)
	<i>Heteropneustes fossilis</i>	8	0.10-0.39	27.9	6.66	10.90	0.29	72	(Farhat and Khan, 2014)
	<i>Heteropneustes fossilis</i>	6	0.04-0.54	22.8	4.45	3.59	0.50	56	(Ahmed, 2012)
	<i>Labeo rohita</i>	6	0.22-0.44	25.8	0.36	6.81	0.31	84	(Abidi and Khan, 2010)
	<i>Cirrhinus mrigala</i>	6	0.06-0.56	27.7	0.62	3.77	0.47	56	(Ahmed and Khan, 2005a)
	<i>Catla catla</i>	6	0.10-0.34	27.7	0.60	16.44	0.31	84	(Zehra and Khan, 2015)
	<i>Oncorhynchus mykiss</i>	12	0.13-0.56	15.5	49.72	3.54	0.58	64	(Rodehutscord <i>et al.</i> 1997)
	<i>Oncorhynchus mykiss</i>	5	0.01-0.43	9.0	5.45	4.60	0.24	112	(Poston and Rumsey, 1983)
	<i>Oncorhynchus mykiss</i>	6	0.08-0.60	15.0	13.76	3.82	0.36	84	(Walton <i>et al.</i> 1984a)
Val	<i>Ictalurus punctatus</i>	7	0.40-1.60	26.7	201	2.77	0.61	56	(Wilson <i>et al.</i> 1980)
	<i>Labeo rohita</i>	6	0.75-2.00	26.0	0.16	2.69	0.70	42	(Abidi and Khan, 2004)
	<i>Cirrhinus mrigala</i>	6	0.75-2.00	27.7	0.62	4.13	0.54	56	(Ahmed and Khan, 2006)
	<i>Oncorhynchus mykiss</i>	12	0.62-3.42	15.7	49.36	3.55	0.43	53	(Rodehutscord <i>et al.</i> 1997)
	<i>Oncorhynchus tshawytscha</i>	6	0.65-1.90	10.0	0.67	3.90	0.78	70	(Chance <i>et al.</i> 1964)

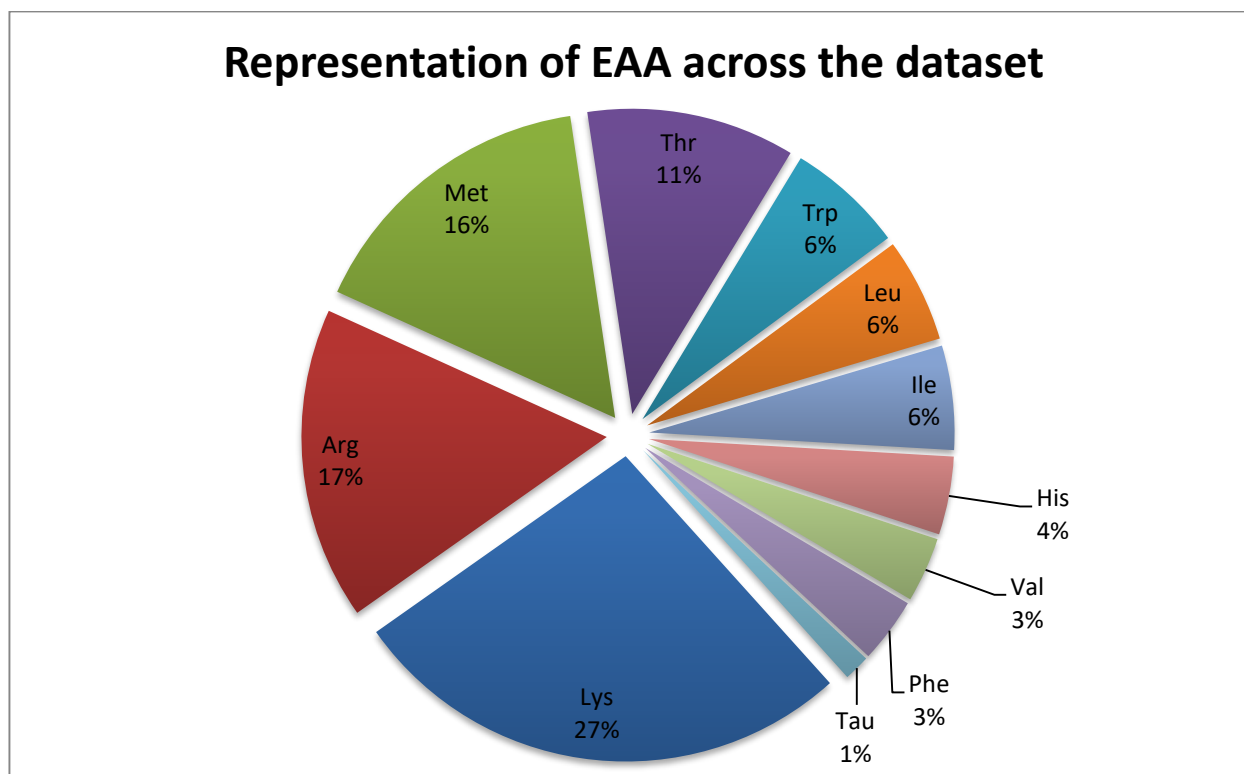


Figure 2: Representation of the eleven EAA in the dataset

In Table 3 we also see the requirements estimated with different variable pairs. While the influence of different response variable can be observed directly, it is meaningless to compare requirements computed with different modes of expression, since they are expressed in different units. Instead, the coefficient of variation (CV) of requirement estimates provides a good indication of the estimate consistency between variable pairs. Regardless of the species and EAA, CVs hover around 22% (median is 15% across the full dataset), which is comparable to the 34% found by Hauler and Carter (2001), but far less than the 100%+ described by Shearer (2000). Bodin *et al.* (2009) found that the choice of response variable impacts the relative lysine requirement in rainbow trout, but not the absolute (i.e. lysine intake) requirement. In our dataset, absolute requirement estimates are indeed numerically closer between response variables, than the relative requirement estimates. However, the variability of the estimates precludes us from establishing statistical significance. By focusing on the requirements for rainbow trout, shows that none of the four mathematical models results in more precise estimates. As well, and contrary to the conclusions of Hauler and Carter (2001) or Bodin *et al.* (2009), we did not observe a significant difference in estimate deviation between the two relative (% diet dry matter and g/MJDE) and the absolute (g ingested/kg MBW/d) modes of

expression. However, the relationship between lysine requirement in rainbow trout and IBW depended on the unit of requirement (Figure 3): there is a significant linear regression when the requirement is expressed as g/kg MBW/d ($P=0.0041$, $R^2=0.482$), but not when expressed as percent of diet dry matter ($P=0.3170$, $R^2=0.077$). Therefore, differences in fish size account for some of the observed deviation, and can be better quantified using an absolute, intake-based mode of expression. Overall, the variability in requirement estimates limits our interpretation of EAA requirements in fish. It also underscores the need to better identify factors governing EAA requirements. This is clearly reflected by the debate over the mode of expression of EAA requirement. The three modes of expression chosen for this meta-analysis represent three, fundamentally different approaches which are based on mutually-exclusive assumptions. Therefore, there is a great need to further investigate the determinants of EAA requirements, and notably their dependence to dietary ingredients or feed intake.

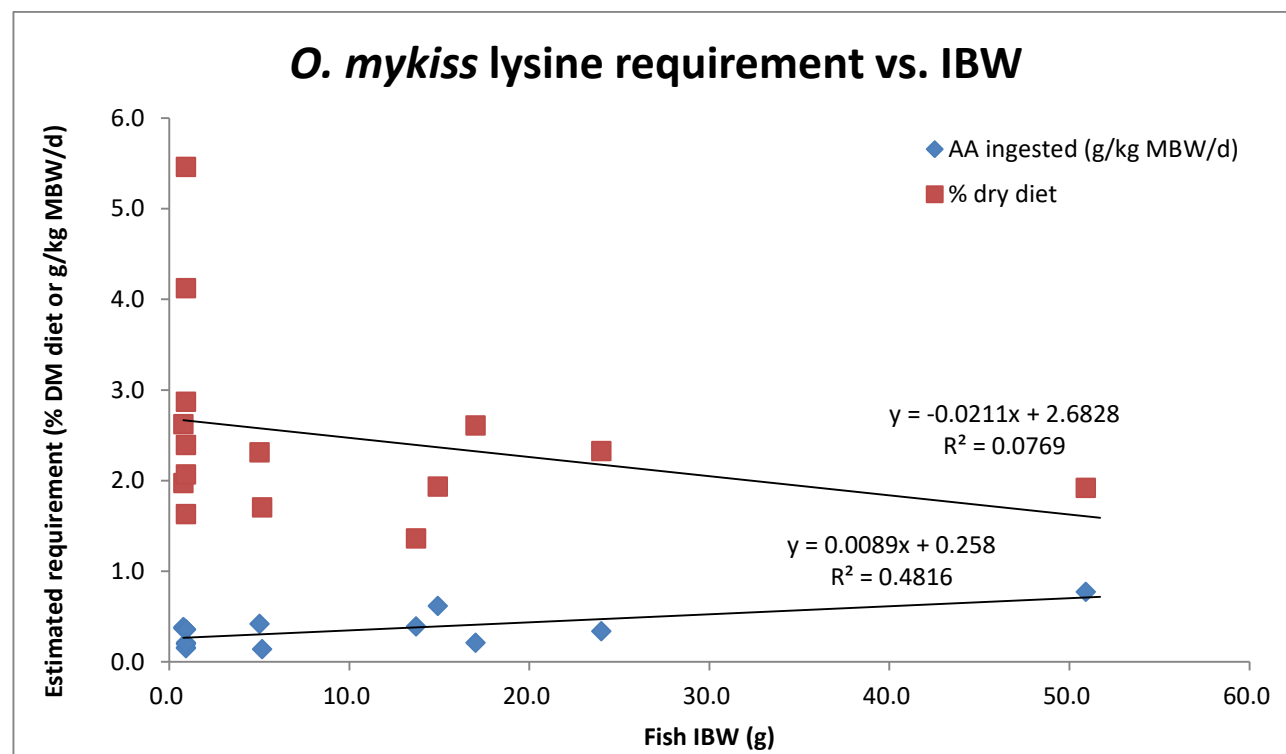


Figure 3: Relationship between initial body weight (IBW, g) and lysine requirement of *O. mykiss* expressed as a percent of diet dry matter or as an ingested amount.

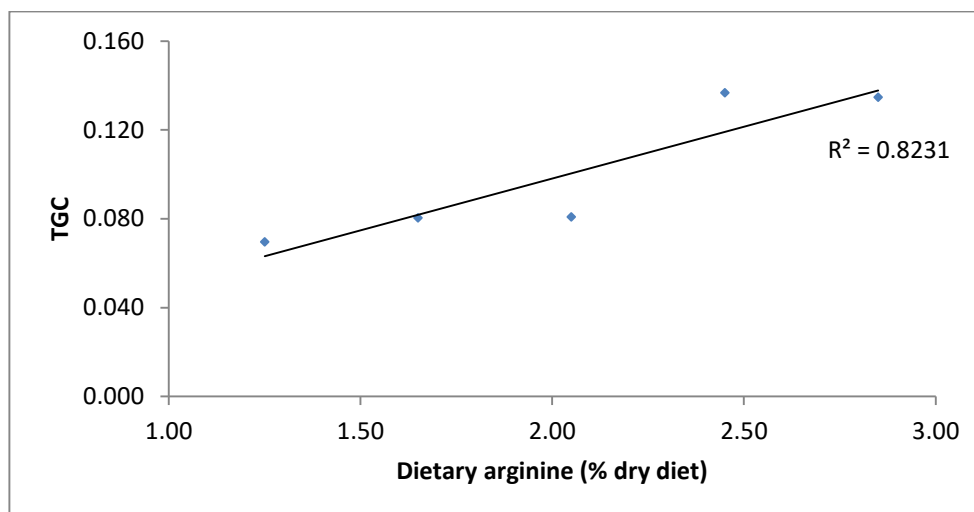


Figure 4: Growth response of turbot (*Psetta maxima*) to graded levels of dietary arginine, illustrating a linear pattern. Adapted from Fournier *et al.* (2003).

3.3 Overall limitations in the dataset

One of the biggest missing pieces of information in the present dataset is digestibility. Indeed, only 15 of the 145 selected studies measured and reported protein digestibility, and none measured the digestibility of individual EAA. Additionally, experimental diets were formulated using a wide range of ingredients. In the selected studies, diets were typically formulated using a mix of natural proteins (fishmeal, soy and corn proteins, etc.) and crystal amino acids. Not only the EAA supplied by these ingredients likely have different digestibility, but there may be relevant interactions among amino acids as well as with other non-protein components. These interactions may affect the availability of EAA to the animal, hence the apparent EAA requirement. For example, phytate is known to bind to amino acids – especially basic amino acids such as lysine, histidine and arginine – which limits their bioavailability to the animal (Chowdhury *et al.*, 2012; Selle *et al.*, 2006; Storebakken *et al.*, 2000). Therefore, data from studies using phytate-rich ingredients such as plant proteins should be carefully interpreted.

Table 3: Estimates of essential amino acid requirements computed using the quadratic model (QM)

Estimates of requirements						
Unit of requirement estimate	Percent of diet dry matter		g/MJ DE		g ingested AA/kg MBW/d	
Growth variable	g/kg MBW/d	TGC	g/kg MBW/d	TGC	g/kg MBW/d	TGC
Arg						
<i>O. kisutch</i>	1.83 ± 69.9%	1.94 ± 60.9%	1.17 ± 68.5%	1.24 ± 59.8%	0.09 ± 8.5%	0.10 ± 1.2%
<i>O. mykiss</i>	1.66 ± 19.0%	1.69 ± 19.0%	0.95 ± 15.5%	0.97 ± 15.4%	0.23 ± 13.0%	0.23 ± 13.3%
<i>S. salar</i>	1.86 ± 4.8%	1.87 ± 3.5%	0.93 ± 10.3%	0.94 ± 11.6 %	0.11 ± 51.4%	0.11 ± 52.9%
Ile						
<i>O. tshawytscha</i>	0.97 ± 6.3%	0.98 ± 7.0%	0.65 ± 6.2%	0.66 ± 7.0%	0.02 ± 3.7%	0.02 ± 4.8%
Lys						
<i>I. punctatus</i>	1.53 ± 0.8%	1.55 ± 14.9%	1.44 ± 1.0%	1.46 ± 6.3%	0.35 ± 15.1%	0.36 ± 6.1%
<i>O. mykiss</i>	2.02 ± 24.0%	2.04 ± 27.9%	1.02 ± 25.7%	1.03 ± 36.6%	0.25 ± 26.9%	0.25 ± 36.4%
<i>S. salar</i>	1.95 ± 46.9%	1.96 ± 49.7%	0.97 ± 39.2%	0.98 ± 41.1%	0.10 ± 51.4%	0.11 ± 52.1%
Met						
<i>Hybrid striped bass</i>	0.96 ± 21.1%	0.97 ± 21.1%	0.75 ± 21.1%	0.76 ± 21.5%	0.24 ± 52.8%	0.25 ± 54.2%
<i>I. punctatus</i>	0.76 ± 21.1%	0.69 ± 40.0%	0.74 ± 17.7%	0.66 ± 36.4%	0.13 ± 46.1%	0.12 ± 61.4%
<i>O. mykiss</i>	0.61 ± 10.5%	0.63 ± 11.4%	0.37 ± 5.2%	0.37 ± 4.9%	0.09 ± 11.9%	0.09 ± 11.4%
Thr						
<i>Hybrid striped bass</i>	0.92 ± 0.5%	0.94 ± 0.0%	0.72 ± 0.8%	0.74 ± 1.0%	0.08 ± 9.6%	0.08 ± 9.0%
<i>O. mykiss</i>	1.26 ± 14.0%	1.27 ± 14.0%	0.70 ± 6.4%	0.70 ± 6.1%	0.16 ± 20.5%	0.16 ± 20.6%
Trp						
<i>O. mykiss</i>	0.29 ± 16.4%	0.29 ± 16.2%	0.17 ± 11.3%	0.17 ± 11.1%	0.03 ± 30.7%	0.03 ± 31.1%

Values are estimates of requirement in the indicated unit, ± the coefficient of variation.

Table 4: Comparisons of lysine requirement estimates in rainbow trout *O. mykiss*, depending on choice of variables and mathematical model

Estimates of requirements (\pm coefficient of variation)						
Unit of requirement estimate	Percent of diet dry matter		g/MJ DE		g ingested AA/kg MBW/d	
Growth variable	g/kg MBW/d	TGC	g/kg MBW/d	TGC	g/kg MBW/d	TGC
Broken line	1.66 \pm 1.73 \pm		0.81 \pm 0.94 \pm		0.19 \pm 0.18 \pm	
	21.6%	22.5%	21.4%	43.5%	30.9%	42.0%
	(n=14)	(n=12)	(n=13)	(n=9)	(n=14)	(n=12)
Broken quadratic	1.69 \pm 1.72 \pm		0.86 \pm 0.88 \pm		0.20 \pm 0.21 \pm	
	17.1%	20.2%	17.6%	33.4%	19.7%	33.2%
	(n=11)	(n=11)	(n=11)	(n=11)	(n=11)	(n=11)
Quadratic	1.83 \pm 1.86 \pm		0.96 \pm 0.97 \pm		0.22 \pm 0.22 \pm	
	22.9%	28.0%	22.8%	38.3%	28.3%	38.0%
	(n=12)	(n=12)	(n=12)	(n=12)	(n=13)	(n=13)
Saturation kinetic	1.99 \pm 2.10 \pm		1.05 \pm 1.13 \pm		0.28 \pm 0.30 \pm	
	17.9%	12.4%	20.5%	22.2%	15.5%	24.1%
	(n=8)	(n=6)	(n=7)	(n=6)	(n=5)	(n=6)

The starting body weight of fish also greatly varies between studies, from 0.16g to 662g, although 60% of the studies started with fish between 0.5 and 25g. However, nutrient utilization is known to change with fish size, including in early juvenile stages (Dumas *et al.* 2007). Therefore, considering EAA requirements across growth stanza likely introduces a source of variation in the requirement estimates.

Finally, only a minority of studies reported final proximate analysis of the fish, or protein deposition results. This precluded us from including variables such as protein retention in our analysis, which have also been debated as a possible way to express EAA requirements.

3.4 Choice of mathematical model and experimental design

Although the choice of mathematical model does not improve the variability of requirement estimates, there were significant differences in performances and quality of fit. The BLM, BQM and QM always converged, while the SKM failed to do so in 19.9% of the selected studies, all variable pairs combined. It should be noted here that a converged model does not sufficient to produce an accurate and/or precise estimation of the requirement. Table 5 shows which parameters of experimental design the models are most sensitive to, and the quality of the outcome beyond model convergence. Both BLM and SKM are sensitive to the number of experimental diets. For instance, each additional diet in the experimental design will make the BLM 0.23 times as likely to produce a minimum instead of a maximum (or 4.35 times as likely to produce a maximum, $p=0.0028$). The SKM model, although closer to biological reality, demands careful experimental design. Although a low number of diets will not significantly increase the risk of failed fit, each additional diet will make the SKM 0.89 times as likely to result in an estimate outside the experimental range ($p=0.0059$). This is problematic, and an “outside” fit should not be considered accurate.

Although fish growth was used as a screening criteria for constituting the working dataset, the maxFBW/IBW ratio still significantly affected how the QM and BQM models fitted the data (Table 5). High weight gain in the best (presumably replete) treatments during the experiment resulted in a reduced risk of producing an estimate outside the experimental range – odds ratio of 0.63 ($p=0.0111$) and 0.71 ($p=0.0084$) for the BQM and QM, respectively. In other words, sufficient weight gain during the experiment contributes to estimates within the experimental range, and in that sense, contribute to the accuracy of the estimates. More surprisingly, the BQM also has a significant tendency to fit a minimum (i.e., negative slope of the quadratic segment) at high maxFBW/IBW

values. Upon closer inspection of such studies, fish growth response to the graded EAA levels is always quite linear, as illustrated in Figure 4. However, all models are based on the law of “diminishing returns”, and thus are best suited for situations that illustrate such a pattern. In 50% of failed BQM cases (and 97% of failed BLM), the model could not fit one of the 2 lines. This highlights that models are sensitive to the presence of both the initial ascending portion of the curve, and the following plateau. Models’ sensitivity to the former is well illustrated by the ratio between the lowest and highest fish weight at the end of the study (minFBW/maxFBW).

Table 5: Odds ratio of maxFBW/IBW, minFBW/maxFBW, and number of diets affecting the fit outcome of the requirement models.

Parameter Outcome	Broken Model	Quadratic	Broken-Line Model	Quadratic Model	Saturation Kinetic Model
maxFBW / IBW ratio					
Failed fit	NS		NS	NS	NS
Minimum	1.15		NS	NS	
No break point	NS		NS		
Outside estimate	0.63		NS	0.71	NS
minFBW / maxFBW ratio					
Failed fit	NS		NS	NS	30.73
Minimum	NS		83.75	NS	
No break point	106.40		26.28		
Outside estimate	NS		0.31	17.71	3.20
Number of Diets					
Failed fit	NS		NS	NS	NS
Minimum	NS		0.23	NS	
No break point	NS		NS		
Outside estimate	NS		NS	NS	0.89

Note: values odds ratio of an outcome occurring vs. a converged fit resulting in an estimate within the experimental range. Minimum: the model has a minimum instead of a maximum; No break point: the breaking point could not be

determined; Failed fit: the model failed to converge; Outside estimate: the estimated requirement is outside of experimental range. NS: odd ratio non-significant different from 1; Empty cells indicate that this outcome is non-applicable to the model.

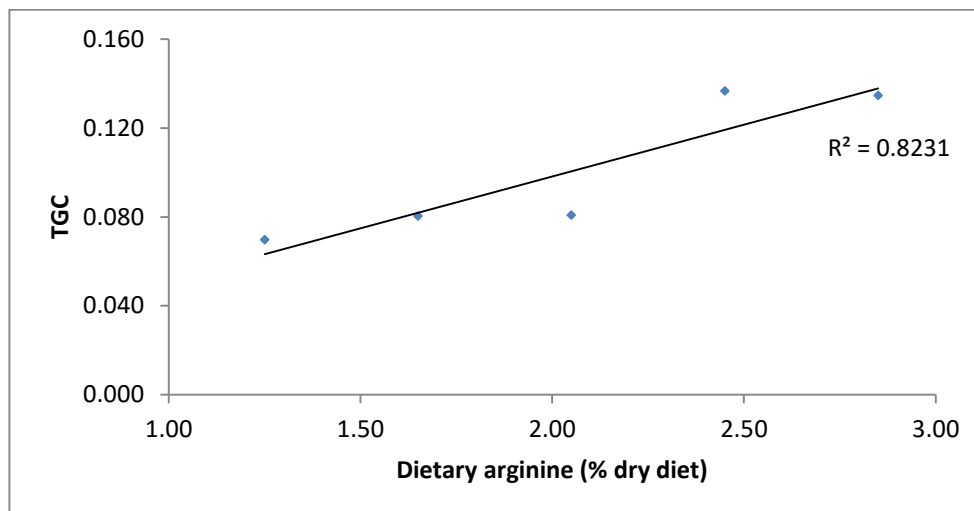


Figure 5: Growth response of turbot (*Psetta maxima*) to graded levels of dietary arginine, illustrating a linear pattern. Adapted from Fournier *et al.* (2003).

The minFBW/maxFBW was by far the most impactful metric regarding fit performance and quality in all models. If one could imagine a 1 unit increase in minFBW/maxFBW ratio, the BQM and the BLM would be 106.40 ($p=0.0307$) and 26.28 ($p=0.0319$) times as likely to fail in finding a breaking point, respectively. The BLM would also be 83.75 times as likely to fit a first line with a negative slope ($p=0.0392$). Finally, the SKM would be 3.20 ($p=0.0170$) and 30.73 ($p<0.0001$) times as likely to find an estimate outside the experimental range and fail altogether, respectively. Evidently, studies failing to maximize the difference in final average weights between treatments at the end of the experiment are in great risk of not being able to accurately estimate the requirement. This occurs when the basal diet is not deficient enough and/or amino acid contents in other experimental diets

do not reach a high enough level to produce a clear plateau. Consequently, particular attention should be given to diet formulations to create such a pattern.

The above discussion highlights factors which nutritionists should carefully consider when developing the experimental design and formulating experimental diets. Our results show that none of the four models result in a significantly improved precision of the requirement estimate. The BLM has been a model of choice for decades, due to its simplicity and ease of fit, especially at a time when computers were not as ubiquitous. However, the limitations of the BLM have been brought to light (Hernandez-Llamas, 2009) and other models have since been suggested. However, no model can be a priori assumed to be superior in all situations, and a careful model selection procedure should be implemented for each experiment as part of the statistical evaluation of the results. Several methods exist for this, although the Akaike Information Criterion (AIC) method (Akaike, 1974; Arnold, 2010), which balances goodness of fit and parsimony of the models, is practical and provides guidance to the nutritionist in his/her choice of model selection (Salze *et al.* 2017).

4 Conclusions and recommendations

The standardization of the current body of scientific literature, as presently conducted, did not lead to clearer, more precise requirement estimates. However, it did highlight some important points. First, the quality of published studies is highly variable: less than half of the relevant studies were deemed suitable for our final data-set. The main reasons for these rejections include poor growth and a lack of reported information such as diet moisture content.

Future investigations of EAA requirements should focus on non-salmonid species, different life stages, and less-studied EAA such as taurine, phenylalanine, leucine, or histidine in order to remedy the knowledge fragmentation. Trials should use no fewer than 6 experimental diets, more if available facilities and resources allow. The use of the BLM should be avoided, as it has now been clearly demonstrated that it underestimates the requirement. Choice of mathematical model should then follow a rational evaluation of fit quality. SKM, QM, or BQM are all valid models, and none should

be *a priori* assumed inherently superior to another. In addition, it is important that the number and range of graded dietary levels of the test EAA are chosen so that the ascending and plateau segments of the curve can be clearly established with the requirement falling approximately in the middle. Finally, the effective loss of it knowledge due to unreported information is very counterproductive. It is therefore most critical that fish nutritionists agree on a standard and systematic system to report and capture results of such trial.

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Diversification of Fads2 in Finfish Species: Implications for Aquaculture

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Abstract

The capabilities for biosynthesis of long-chain ($\geq C_{20}$) polyunsaturated fatty acid (LC-PUFA) of farmed fish have been extensively studied in order to determine qualitative requirements for dietary essential fatty acids and to ensure high levels of omega-3 LC-PUFA in the farmed products for human consumption. Although LC-PUFA biosynthesis comprises multiple steps catalyzed by several enzymes, rate-limiting reactions in the pathways are controlled by fatty acid desaturases (Fads), enzymes introducing new double bonds into fatty acyl chains. The repertoire of Fads-encoding genes varies among vertebrates. Mammals have two FADS with known roles in the LC-PUFA biosynthetic pathways, namely FADS1 with $\Delta 5$ desaturase activity and FADS2 with $\Delta 6$ activity. Interestingly, teleosts, the fish group which most farmed species belong to, appear to have lost *fads1* during evolution and therefore Fads2 is the sole enzyme able to account for the desaturation reactions in the LC-PUFA pathway in teleosts. Unlike mammals though, functions of teleost Fads2 have diversified remarkably as a result of species-specific evolutionary history and environmental factors including habitat (marine vs freshwater), trophic level and ecology. This paper reviews the recent progress made on molecular aspects underlying the functional diversity of Fads2 characterized so far from finfish species. Specifically, we discuss the potential implications that Fads2 functions have for the ability of fish species to efficiently utilize dietary fatty acids when fed on vegetable oil-based feeds. In addition, current developing technologies including genetic approaches (e.g. transgenesis) to improve the LC-PUFA biosynthetic capability of fish are discussed.

Keywords: Fads2, Finfish, Aquaculture

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Introduction

Fish and seafood are universally recognized as important components of a healthy diet as they supply high quality, easily digested protein, essential micronutrients including the minerals selenium and iodine, and vitamins (Tacon & Metian, 2013). However, the nutrients most associated with the beneficial effects of eating fish are the n-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Lands, 2014). These key conditionally-essential fatty acids have been the most studied and have key roles in neural development, and beneficial effects in cardiovascular and inflammatory diseases and some cancers (Calder, 2014). The physiological, biochemical and molecular mechanisms underpinning the critical roles of these “omega-3” fatty acids in human health are increasingly being elucidated and understood (Calder, 2015). Our understanding of the beneficial effects of dietary EPA and DHA on human health have been largely based on two main lines of evidence, epidemiological studies and randomized controlled (intervention) trials, although laboratory studies investigating biochemical and molecular mechanisms have also provided mechanistic support to these *in vivo* approaches (Gil *et al.* 2012; Calder, 2015). Based on all the evidence, many recommendations for EPA and DHA intake for humans have been produced by a large number of global and national health agencies and associations, and government bodies with those of over 50 organizations compiled recently by the Global Organization for EPA and DHA Omega 3s (GOED, 2014). While recommended levels vary between 250 and 1000 mg, the International Society for the Study of Fatty Acids and Lipids recommend a daily intake of 500 mg of n-3 LC-PUFA for optimum cardiovascular health (ISSFAL, 2004). Projecting this to a world population of 7 billion, this amounts to a total annual requirement for over 1.25 million metric tonnes (mt) of n-3 LC-PUFA and, as global supply cannot meet this level of requirement, there is a large gap between supply and demand (Naylor *et al.* 2009; Salem

and Eggersdorfer, 2015; Tocher, 2015).

Marine microalgae and microbes are the primary producers of the vast majority of n-3 LC-PUFA (Harwood & Guschina, 2009), which consequently accumulate in the marine food web, underpinning why fish and seafood are the predominant sources of these nutrients in the diet (Tur *et al.* 2012; Sprague *et al.* 2017a). Indeed, consuming at least two portions of fish per week, of which one should be oily, is advised by global health authorities as a means of achieving the recommended daily intake of EPA+DHA in order to protect against cardiovascular and inflammatory diseases, among other health benefits (GOED, 2014). However, global fisheries are at, or beyond, exploitable limits and cannot satisfy the growing demand for fish and seafood (Worm *et al.* 2009). Consequently, aquaculture, which has been growing at over 6 % per year for around two decades, is filling the gap such that over 50 % of global fish and seafood is now farmed (FAO, 2016). Interestingly, high levels of n-3 LC-PUFA in farmed fish and shrimp were only assured by formulating feeds with high levels of fishmeal and, especially, fish oil, paradoxically themselves finite and limited marine resources derived from wild fisheries (Tacon & Metian, 2008; Shepherd & Jackson, 2013; NRC, 2011). Therefore, for aquaculture to continue to expand, alternatives to dietary fishmeal and oil have been sought, with plant meals and vegetable oils currently the only viable, sustainable alternatives (Turchini *et al.* 2011; Shepherd *et al.* 2017). However, these alternative ingredients do not contain n-3 LC-PUFA as the biosynthetic pathway for their production is not present in terrestrial plants (Harwood, 2005), and this has presented some major challenges for aquaculture.

Vegetable oils can contain high levels of the short-chain (<C₂₀) PUFA, α -linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6), although these fatty acids have no major functional roles in most fish other than as precursors of the highly biologically active LC-PUFA, EPA, DHA and arachidonic acid (ARA, 20:4n-6) (Tocher, 2003). While PUFA in general are essential dietary components for all fish, the specific essential fatty acids (EFA)

vary between species, with LNA and LOA able to satisfy EFA requirements in many freshwater and salmonid fish, whereas only the LC-PUFA themselves can satisfy EFA requirements in most marine teleosts (Tocher, 2010). However, in almost all species, dietary LC-PUFA support better growth performance than C₁₈ PUFA (Tocher, 2010). Therefore, the critical importance of dietary LC-PUFA for optimal growth, development and health of all vertebrates including fish (Calder, 2014, 2015; Tocher, 2003, 2010) means that dietary fishmeal and fish oil replacement, with consequent reduction in dietary n-3 LC-PUFA, while entirely necessary, has potentially impacted aquaculture production and fish health (Tocher & Glencross, 2015). In addition, it has also resulted in levels of n-3 LC-PUFA in farmed fish declining in recent years with the associated impact on product/nutritional quality for human consumers (Sprague *et al.* 2016; De Roos *et al.* 2017). This has stimulated much research into mitigating and reversing this decline, and to increasing the global supply of EPA and DHA (Tocher, 2015; Sprague *et al.* 2017b). It is in this context that the considerable research effort into elucidating pathways of fatty acid metabolism in fish has been based with the overarching hypothesis that understanding the molecular basis of LC-PUFA biosynthesis and regulation will enable the pathway to be optimized to promote efficient and effective use of plant-based dietary ingredients in aquafeeds, and n-3 LC-PUFA contents of farmed fish to be restored (Leaver *et al.* 2008; Torstensen & Tocher, 2011).

Biosynthetic pathway of long-chain polyunsaturated fatty acids

The biosynthetic pathways of LC-PUFA in vertebrates including fish is restricted to conversion of C₁₈ PUFA precursors including LA and ALA supplied in the diet. Two types of key enzymes, namely fatty acid desaturases (Fads) and elongation of very long-chain fatty acids (Elovl) proteins (commonly refer to as “elongases”), mediate the rate-limiting reactions in LC-PUFA biosynthetic pathways (Figure 1).

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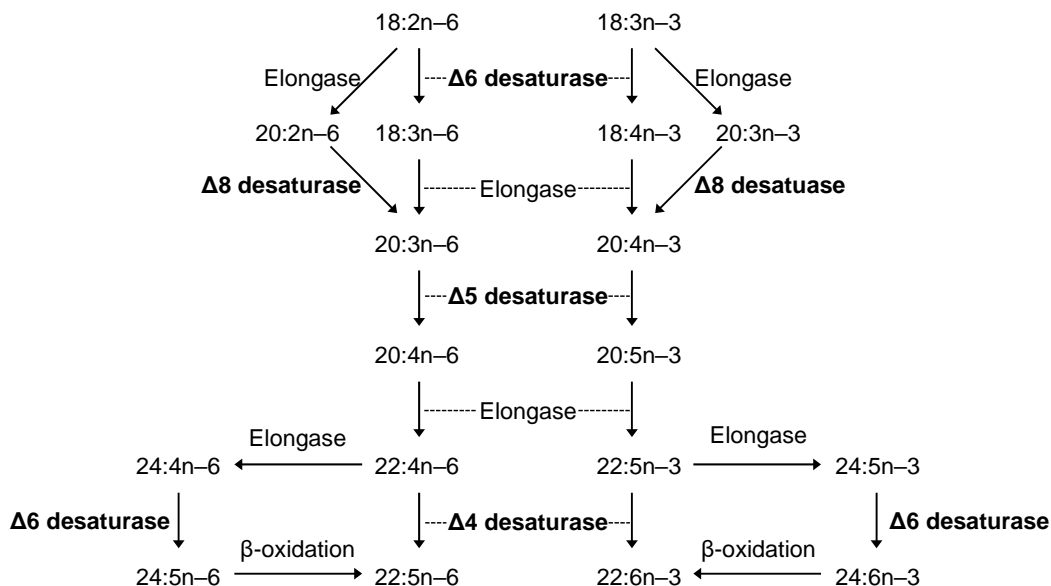


Figure 1. Complete biosynthetic pathways of long-chain polyunsaturated fatty acids (LC-PUFA) from the C_{18} precursors, namely $18:2n-6$ and $18:3n-3$ in vertebrates.

The Fads and Elovl enzymes involved in specific reactions of these pathways act on both the n-3 and n-6 series fatty acids, with n-3 fatty acids generally being preferred substrates (Castro *et al.* 2016). Using n-3 PUFA as an example, biosynthesis of EPA from ALA requires a $\Delta 6$ desaturation to produce $18:4n-3$, which is then elongated to $20:4n-3$. The latter goes through a $\Delta 5$ desaturation resulting in the production of EPA. An alternative pathway involves initial elongation of ALA to $20:3n-3$, which is then converted to $20:4n-3$ via $\Delta 8$ desaturation. As in the previous pathway, $20:4n-3$ can be converted to EPA by the action of a $\Delta 5$ desaturase. In mammals, the production of DHA from EPA appears to proceed through the so-called “Sprecher pathway”, a metabolic route that involves two consecutive elongations from EPA to produce $24:5n-3$, the latter then being $\Delta 6$ desaturated to $24:6n-3$ (Sprecher, 2000). While all the described reactions so far described take place in the endoplasmic reticulum (ER), a partial β -oxidation reaction of $24:6n-3$ to DHA occurs in the peroxisomes and hence DHA biosynthesis through the Sprecher pathway is regarded as a more complicated route than the

“ $\Delta 4$ pathway”, an alternative route for DHA biosynthesis proven to potentially operate in a number of fish species (Oboh *et al.* 2017). The presence of Fads enzymes enabling the $\Delta 4$ pathway in fish will be extensively covered in the following sections, although it should be noted that these pathways were recently proved to also potentially operate in human cells (Park *et al.* 2015).

Fads enzymes catalyze the introduction of a double bond (unsaturation) between an existing double bond and the carboxylic group at the terminus of the fatty acid substrate and, therefore, are known as “front-end” desaturases (Castro *et al.* 2016). The repertoire of Fads varies among vertebrates (Castro *et al.* 2012). Mammals possess three Fads-like enzymes termed FADS1, FADS2 and FADS3. FADS1 is a $\Delta 5$ desaturase, whereas FADS2 is a $\Delta 6$ desaturase with the ability to utilize both C_{18} substrates at the beginning of the pathways (Figure 1) and C_{24} PUFA including 24:5n-3 involved DHA biosynthesis via the Sprecher pathway (Sprecher, 2000). Further activities of mammalian FADS2 include the above mentioned $\Delta 8$ desaturation (Park *et al.* 2009) and, at least in a cell culture system, $\Delta 4$ activity (Park *et al.* 2015). Although the precise function(s) of FADS3 in LC-PUFA biosynthesis remain largely unknown, a recent study using a *Fads3* knock-out murine model resulted in reduced levels of DHA in brain and a higher ratio of 22:5n-3 to DHA in liver compared to wild-type, which suggested that Fads3 may enhance liver-mediated DHA synthesis to support brain accretion (Zhang *et al.* 2017). Although being beyond the scope of the present review, it is important to note that, in addition to Fads, Elovl (elongases) also play major roles in LC-PUFA biosynthesis. Briefly, Elovl enzymes catalyze the first and rate-limiting condensation step in the reactions that result in the 2-carbon elongation of fatty acids (Jakobsson *et al.* 2006). Seven members of the Elovl protein family (Elovl1-7) have been described in vertebrates (Guillou *et al.* 2010). While Elovl1, Elovl3, Elovl6 and Elovl7 have saturated or monounsaturated fatty acids as preferred substrates, Elovl2, Elovl4 and Elovl5 elongate PUFA and therefore play important roles in LC-PUFA biosynthesis (Jakobsson *et al.* 2006;

Guillou *et al.* 2010). Indeed, these enzymes have been the focus of investigation in a wide range of fish species and the repertoire and functions of Elovl enzymes in fish have been reviewed previously (Monroig *et al.* 2011a; Castro *et al.* 2016).

Diversification of Fads2 desaturase activity in fish

There is strong evidence supporting that virtually all teleosts have lost the *fads1* gene during evolution, although both *fads1* and *fads2* genes could still be identified in cartilaginous fish such as the smaller spotted catshark *Scyliorhinus canicula* (Castro *et al.* 2012). Loss of *fads1* in teleosts has been accompanied by the expansion of the *fads2* gene and the diversification of the enzymes' functions. These phenomena have been elucidated by cloning and functional characterization of *fads2* genes from a wide range of phylogenetically diverse groups of teleosts from different habitats (freshwater, diadromous, catadromous and marine) and trophic levels (from herbivores to top carnivores). We herein provide an updated list of all Fads2 cDNAs that have been cloned and functionally characterized from teleosts. As shown in Table 1, Fads2 from most of species showed $\Delta 6$ desaturase activity, consistent with the functions of the mammalian FADS2 (Guillou *et al.* 2010). Moreover, a comprehensive study investigating the $\Delta 8$ desaturase capability of teleost Fads2 confirmed that such desaturation ability occurred in all teleost Fads2 enzymes tested (Monroig *et al.* 2011b), the list now expanded to include other Fads2 from meagre *Argyrosomus regius*, orange-spotted grouper *Epinephelus coioides*, and nibe croaker *Nibea mitsukurii* (Monroig *et al.* 2013; Li *et al.* 2014; Kabeya *et al.* 2015). Searches in genome assemblies from several commercially important marine species (e.g. Atlantic cod *Gadus morhua* and European seabass *Dicentrarchus labrax*) confirmed that only one copy of *fads2* exists in the genome indicating that desaturation abilities were restricted to $\Delta 6$ and $\Delta 8$. These findings were in agreement with the commonly accepted view that marine species have limited LC-PUFA biosynthesis capabilities (Tocher,

2010), this being why most of commercially important marine species strictly require the supply of preformed LC-PUFA, particularly EPA and DHA, in their diet (Tocher, 2010). This situation was aggravated even further in species such as the two pufferfish species *Takifugu rubripes* and *Tetraodon nigroviridis*, which completely lack *fads*-like genes in their genome (Castro *et al.* 2012).

Table 1. Functionally characterized Fads2 in teleost. All activities were determined by the yeast heterologous expression.

Species	Common name	Activities	References
<i>Danio rerio</i>	Zebrafish	$\Delta 6, \Delta 5, \Delta 8$	Hastings <i>et al.</i> , 2001; Monroig <i>et al.</i> , 2011
<i>Oncorhynchus mykiss</i>	Rainbow trout	$\Delta 6, \Delta 8$	Zheng <i>et al.</i> , 2004; Monroig <i>et al.</i> , 2011
<i>O. mykiss</i>		$\Delta 5$	Hamid <i>et al.</i> , 2016
<i>Cyprinus carpio</i>	Common carp	$\Delta 6$	Zheng <i>et al.</i> , 2004
<i>Sparus aurata</i>	Gilthead sea bream	$\Delta 6, \Delta 8$	Zheng <i>et al.</i> , 2004; Monroig <i>et al.</i> , 2011
<i>Scophthalmus maximus</i>	Turbot	$\Delta 6, \Delta 8$	Zheng <i>et al.</i> , 2004; Monroig <i>et al.</i> , 2011
* <i>Salmo salar</i> ($\Delta 5$ Fad)	Atlantic salmon	$\Delta 5$	Hastings <i>et al.</i> , 2005
<i>S. salar</i> ($\Delta 6$ Fad_a)		$\Delta 6$	Zheng <i>et al.</i> , 2005
<i>S. salar</i> ($\Delta 6$ Fad_b)		$\Delta 6, \Delta 8$	Monroig <i>et al.</i> , 2010, 2011
<i>S. salar</i> ($\Delta 6$ Fad_c)		$\Delta 6, \Delta 8$	Monroig <i>et al.</i> , 2010, 2011
<i>Gadus morhua</i>	Atlantic cod	$\Delta 6, \Delta 8$	Tocher <i>et al.</i> , 2006; Monroig <i>et al.</i> , 2011
<i>Rachycentron canadum</i>	Cobia	$\Delta 6, \Delta 8$	Zheng <i>et al.</i> , 2009; Monroig <i>et al.</i> , 2011
<i>Dicentrarchus labrax</i>	European sea bass	$\Delta 6$	González-Rovira <i>et al.</i> , 2009; Santigosa <i>et al.</i> , 2011
<i>Lates calcarifer</i>	Barramundi	$\Delta 6$	Mohd-Yusof <i>et al.</i> , 2010
* <i>Siganus canaliculatus</i> (Fad1)	Rabbitfish	$\Delta 6, \Delta 5, \Delta 8$	Li <i>et al.</i> , 2010; Monroig <i>et al.</i> 2011
<i>S. canaliculatus</i> (Fad2)		$\Delta 4, \Delta 8$	Li <i>et al.</i> , 2010; Monroig <i>et al.</i> 2011
<i>Acanthopagrus schlegelii</i>	Black seabream	$\Delta 6$	Kim <i>et al.</i> , 2011
<i>Thunnus thynnus</i>	Northern bluefin tuna	$\Delta 6$	Morais <i>et al.</i> , 2011
<i>Solea senegalensis</i>	Senegalese sole	$\Delta 4$	Morais <i>et al.</i> , 2012
<i>Oreochromis niloticus</i>	Nile tilapia	$\Delta 6, \Delta 5$	Tanomman <i>et al.</i> , 2013
<i>O. niloticus</i>		$\Delta 4$	Oboh <i>et al.</i> , 2017
<i>Argyrosomus regius</i>	Meagre	$\Delta 6, \Delta 8$	Monroig <i>et al.</i> , 2013
* <i>Chirostoma estor</i> (Fads2a)	Pike silverside	$\Delta 4$	Fonseca-Madrigal <i>et al.</i> , 2014
<i>C. estor</i> (Fads2b)		$\Delta 6, \Delta 5, \Delta 8$	Fonseca-Madrigal <i>et al.</i> , 2014
<i>Anguilla japonica</i>	Japanese eel	$\Delta 6, \Delta 8$	Wang <i>et al.</i> , 2014

<i>Epinephelus coioides</i>	Orange spotted grouper	$\Delta 6, \Delta 8$	Li <i>et al.</i> , 2014
<i>Scatophagus argus</i>	Spotted scat	$\Delta 6$	Xie <i>et al.</i> , 2014
<i>Nibea mitsukurii</i>	Nibe croaker	$\Delta 6, \Delta 8$	Kabeya <i>et al.</i> , 2015
<i>Channa striata</i>	Striped snakehead	$\Delta 4, \Delta 5$	Kuah <i>et al.</i> , 2015
<i>C. striata</i>		$\Delta 6, \Delta 5$	Kuah <i>et al.</i> , 2016
<i>Clarias gariepinus</i>	African catfish	$\Delta 6, \Delta 5$	Oboh <i>et al.</i> , 2016
<i>Perca fluviatilis</i>	European perch	$\Delta 6$	Geay <i>et al.</i> , 2016
<i>Arapaima gigas</i>	Arapaima	$\Delta 6$	Lopes-Marques <i>et al.</i> , 2017
<i>Oryzias latipes</i>	Japanese medaka	$\Delta 4$	Oboh <i>et al.</i> , 2017
<i>Nibea coibor</i>	Chu's croaker	$\Delta 6$	Huang <i>et al.</i> , 2017

*gene names referring to the corresponding publications in brackets.

Acquisition of alternative regioselectivities has occurred within teleost *fads2* (Fonseca-Madrugal *et al.* 2014; Castro *et al.* 2016). Indeed, zebrafish *Danio rerio* Fads2, the first functionally characterized Fads2 in a teleost, showed dual $\Delta 6$ and $\Delta 5$ desaturase activities (Hastings *et al.* 2001). Similarly, dual $\Delta 6\Delta 5$ desaturase activity has been reported in other teleost Fads2 including those from the rabbitfish *Siganus canaliculatus* (Li *et al.* 2010), Nile tilapia *Oreochromis niloticus* (Tanomman *et al.* 2013), pike silverside *Chirostoma estor* (Fonseca-Madrugal *et al.* 2014), African catfish *Clarias gariepinus* (Oboh *et al.* 2016) and striped snakehead *Channa striata* (Kuah *et al.* 2016) (Table 1). Interestingly, a recent study demonstrated that an Atlantic salmon *Salmo salar* desaturase, initially characterized as a monofunctional $\Delta 5$ desaturase (Hastings *et al.* 2005), also possessed $\Delta 6$ desaturase activity (Oboh *et al.* 2017). This result leaves the rainbow trout $\Delta 5$ desaturase as the only essentially monofunctional $\Delta 5$ Fads2 desaturase (Hamid *et al.* 2015). The distribution of dual $\Delta 6\Delta 5$ desaturases from species throughout the entire tree of life of teleosts (Betancur *et al.* 2013) suggested that this is a rather common trait among teleost Fads2. Importantly, the acquisition of $\Delta 5$ desaturase within a Fads2 can partly compensate the loss of Fads1 in some teleosts, allowing them to synthesize EPA and ARA from the corresponding C_{18} PUFA precursors, namely ALA and LA, respectively (Figure 1).

Further cases of diversification of teleost Fads2 functions include the ability of some teleost Fads2 to act as $\Delta 4$ desaturases. Such activity was first discovered in the rabbitfish *Siganus canaliculatus*, at that time representing the first case of a $\Delta 4$ desaturase among vertebrates (Li *et al.* 2010). Further $\Delta 4$ Fads2 have been subsequently discovered in other teleosts (Table 1). It is important to note that, in some species such as rabbitfish, the $\Delta 4$ Fads2 co-exists with another Fads2 with $\Delta 6/\Delta 5$ desaturase activity, enabling all the desaturation reactions required for production of DHA from the precursor ALA. While its herbivorous feeding behavior was hypothesized to account for such a desaturase activity complement in rabbitfish (Li *et al.*, 2010), the same pattern was later discovered in non-herbivoreous teleost species such as the pike silverside (Fonseca-Madrigal *et al.* 2014), striped snakehead (Kuah *et al.* 2015; Kuah *et al.* 2016) and Nile tilapia (Tanomman *et al.* 2013; Oboh *et al.* 2017), suggesting that confounding factors other than trophic level can also drive the functional diversification among teleost Fads2.

Fish can biosynthesize DHA through two possible pathways

As introduced above, Sprecher and co-workers demonstrated that the biosynthesis of DHA in mammals did not appear to proceed through a $\Delta 4$ desaturation from 22:5n-3 but rather 22:5n-3 was elongated to 24:5n-3 before the latter was further desaturated at the $\Delta 6$ position to produce 24:6n-3. Chain shortening of 24:6n-3 to DHA was achieved in the peroxisomes and therefore the Sprecher pathway involved translocation of fatty acids from endoplasmic reticulum (responsible for desaturation and elongation reactions) to peroxisomes where partial β -oxidation to DHA takes place. Although these studies were conducted in rats, the same pathways were accepted to mediate DHA biosynthesis in all vertebrates, and further studies confirmed that the Sprecher pathway was also active in rainbow trout (Buzzi *et al.* 1997; Henderson *et al.* 1998). Later, the zebrafish Fads2, which had been proven to act as

$\Delta 6$ desaturase towards 18:3n-3 (Hastings *et al.* 2001), was demonstrated to effectively desaturate 24:5n-3 to 24:6n-3 (Tocher *et al.* 2003). This study therefore confirmed that the same Fads2 enzyme could operate at both of the distinct $\Delta 6$ desaturation steps of the pathway (Figure 1), one on 18:3n-3 and the other on 24:5n-3. In contrast, Kabeya *et al.* (2015) showed that the $\Delta 6$ Fads2 from Nibe croaker *Nibea mitsukurii* was able to desaturate 18:3n-3 but not 24:5n-3, suggesting that the ability of fish to biosynthesize DHA through the Sprecher pathway varies among species. In order to test this hypothesis, a recent study investigated the prevalence of the Sprecher pathway among teleost fish by determining the $\Delta 6$ activity towards C₂₄ substrates (24:5n-3 and 24:4n-6) by teleost Fads2 desaturases (Obloh *et al.*, 2017). The study concluded that, with the exception of the Nibe croaker $\Delta 6$ Fads2, all non- $\Delta 4$ desaturases had the ability to desaturate C₂₄ PUFA substrates at $\Delta 6$ position and therefore both 24:5n-3 and 24:4n-6 were converted to 24:6n-3 and 24:5n-6, respectively. Importantly, such desaturase capability was demonstrated in Fads2 from species with different evolutionary backgrounds and with different desaturase activities including monofunctional $\Delta 6$ and bifunctional $\Delta 6\Delta 5$ desaturases (Obloh *et al.* 2017). Although none of the $\Delta 4$ Fads2 studied by Obloh *et al.* (2017) were able to desaturate C₂₄ PUFA substrates, some of these $\Delta 4$ desaturases were present in fish species (e.g. rabbitfish and pike silverside) in which a further Fads2 had the ability to desaturate 24:5n-3 to 24:6n-3 as per the Sprecher pathway. Consequently, two pathways for DHA biosynthesis, namely the Sprecher pathway and the $\Delta 4$ pathway, could potentially coexist in some teleost fish species and, if both were functional, this would represent a clear advantage for satisfying DHA requirements through endogenous production from dietary precursors.

It is interesting to note that Obloh *et al.* (2017) further confirmed that the occurrence of the $\Delta 4$ pathway was more widespread than initially believed when the first vertebrate $\Delta 4$ desaturase was discovered in the rabbitfish (Li *et al.* 2010). Using a conserved region containing the YXXN motif responsible for $\Delta 4$ desaturase activity (Lim *et al.* 2014) as a

search query, Oboh *et al.* (2017) identified the presence of putative $\Delta 4$ desaturases in 11 species and confirmed their functions in Nile tilapia and Japanese medaka as described above. The distribution of $\Delta 4$ desaturases within the tree of life of bony fishes suggested that $\Delta 4$ Fads2 appear to be restricted to teleost species within recently emerged lineages, suggesting that the acquisition of the $\Delta 4$ pathway occurred later during the evolution of teleosts, with more basal fish lineages (e.g. eels, carps, catfish, salmonids) having (if any) the Sprecher pathway as the sole route for DHA biosynthesis (Oboh *et al.* 2017).

Utilization of molecular information of LC-PUFA biosynthesis for aquaculture

The rapidly accruing molecular information of the LC-PUFA biosynthetic pathway in fish may enable implementation of new genetics-based approaches to optimize and maximize EPA and DHA levels in farmed species. For instance, genetic selection of fish strains with enhanced ability for LC-PUFA biosynthesis appears as a very promising strategy (Gjedrem, 2000) since the n-3 LC-PUFA content in flesh was confirmed to be a highly heritable trait in Atlantic salmon (Leaver *et al.* 2011). Our expanding knowledge of the genes encoding the fatty acyl desaturases and elongases directly associated with the n-3 LC-PUFA trait may therefore make them appropriate targets for selection when desirable alleles are identified. This could enable the development of fish strains with enhanced ability to thrive on more sustainable plant-based feed formulations. In this respect, wild stocks may represent a valuable genetic resource for improving the n-3 LC-PUFA trait as it was shown recently that land-locked strains of Atlantic salmon, which do not migrate to the sea, have increased LC-PUFA biosynthetic capacity (Betancor *et al.* 2016).

Studies have indicated that endogenous LC-PUFA biosynthesis in fish may be enhanced by “nutritional programming” and that this may involve activation and/or optimization of gene expression (Clarkson *et al.* 2017; Vera *et al.* submitted). The nutritional

programming concept involves exposing an animal to a dietary stimulus early in life that alters that individual metabolically and physiologically such that it becomes adapted and better able to respond to a similar nutritional challenge later in life (Lucas, 1998; Patel & Srinivasan, 2002). Very recently, an early nutritional intervention in Atlantic salmon at first feeding using a diet formulated almost entirely with plant meals and vegetable oils, with only very low levels of EPA and DHA, adapted fish to better utilize these feeds. Thus, fish given this challenging diet at first feeding showed greatly increased retentions of EPA and DHA compared to fish fed a diet with high levels of n-3 LC-PUFA at first feeding (Clarkson *et al.* 2017). Liver gene expression showed an up-regulation of all pathways of intermediary metabolism including LC-PUFA biosynthesis (specifically *fads2* and *elovl5* genes) in fish given the early nutritional stimulus that was consistent with a biochemical and physiological response/adaptation (Vera *et al.*, submitted). While these data also provide some insight, the precise nature of the molecular response is still under investigation and likely involves epigenetic mechanisms (Balasubramanian *et al.* 2016).

Transgenic technology has also been applied to enhance the capacity of LC-PUFA biosynthesis. Initial trials using the model species zebrafish were conducted by introducing genes encoding putative $\Delta 6$ (Alimuddin *et al.* 2005) and $\Delta 5$ *fads2* (Alimuddin *et al.* 2007), and *elovl5* (Alimuddin *et al.* 2008) from masu salmon *Oncorhynchus masou*. The resulting transgenic zebrafish carrying masu salmon putative $\Delta 6$ *fads2* showed a level DHA twice as high in comparison with non-transgenic counterparts (Alimuddin *et al.* 2005). The transgenic zebrafish carrying masu salmon putative $\Delta 6$ *fads2* and *elovl5* also showed higher LC-PUFA levels in comparison with non-transgenic counterparts (Alimuddin *et al.* 2007, 2008). Thereafter, humanized *Caenorhabditis elegans* $\omega 3$ desaturase (*fat1*) and $\Delta 12$ desaturase (*fat2*) were also introduced into zebrafish enabling the transgenic fish to produce PUFA *de novo* (Pang *et al.* 2014). As developing transgenic techniques have become applicable to non-model species, transgenic strains have also been established in commercially important

species, namely the common carp *Cyprinus carpio* (Cheng *et al.* 2014) and the marine Nibe croaker (Kabeya *et al.* 2014, 2016). These studies have the potential, not only to understand molecular and physiological mechanisms underlying LC-PUFA metabolism *in vivo*, but also to generate specific strains of farmed fish that would possibly not require a dietary supply of preformed LC-PUFA for larval and on-growing stages. However, the applicability of these technologies to fish farming is still extremely challenging due, in part, to socio-political issues and food safety regulations.

Conclusions

LC-PUFA, particularly those of the omega-3 series, are essential components of the human diet being primarily sourced from fish. Therefore, as aquaculture has continued to expand, the industry has had to confront the high demand for nutritious (high omega-3 LC-PUFA) fish and seafood products whilst increasing the utilization of non-marine ingredients in aquafeeds. Simultaneously, a number of research lines have been conducted to broaden the knowledge with regards to LC-PUFA biosynthesis in fish in order to understand their ability to utilise dietary fatty acids contained in alternative plant-based ingredients used in aquafeeds. In the present review, we have summarized the extent to which our understanding of teleost Fads2, one of the key enzymes of LC-PUFA biosynthesis, has helped to achieve this aim. In contrast to mammals, teleosts have lost the *fads1* gene ($\Delta 5$ desaturase) but possess different copy numbers of *fads2*. Importantly, rather than remaining as $\Delta 6/\Delta 8$ desaturases as in mammals, teleost fish Fads2 have functionalized as a result of species-specific evolutionary histories and ecological factors including habitat and trophic level. In addition, it is noteworthy that two distinct pathways of DHA biosynthesis, the so-called “Sprecher pathway” and “ $\Delta 4$ pathway” have been confirmed to be spread widely among teleosts. However, since Fads2 regioselectivity could be completely different even within closely related species, the functional characterization of Fads2 will continue to provide valuable insights into LC-PUFA biosynthesis in fish and be vital for practical applications within the aquaculture industry.

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Non-coding RNAs: Uncovering their Potential Relevance in Fish Nutrition

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Abstract

The optimization of industrial production would only be possible with the discovery, identification and characterization of biological processes in which a nutrient or any other factor acts, as well as when their genes and genetic networks revealed. With the advent of Next Generation-Sequencing technologies, the discovery of non-coding RNAs having a key role on the control of a diverse set of biological functions in multicellular organism will allow a deeper knowledge on genes and genetic networks control such processes in farmed fish species. Here, the basics of non-coding RNAs regarding their features, biogenesis and mode of action will be briefly reviewed, while the research works specifically conducted until now on the identification of non-coding RNAs in different farmed fish species, developmental stages and tissues using high throughput technologies will be described and compared. Several non-coding RNAs have been associated with early developmental events, immune response to pathogen infections, sexual differentiation and maturation, and nutrition. While the research on miRNAs is the most abundant, new efforts on the characterization of long non-coding RNAs and PIWI-interacting RNAs profiles provided new insights on how these non-coding RNAs are also involved in fish nutrition. Finally, the future perspectives and considerations on the potential use of non-coding RNAs (mainly those found in circulation) in relevant cultured fish species as new reliable biomarkers of physiological condition will be pointed out.

Keywords: ncRNAs, miRNAs, nutrigenomics, transcriptomics, NGS.

1. Beyond the central dogma: the complex world of non-coding RNAs.

Industrial production optimization would only be possible with the discovery, identification and characterization of biological processes in which a nutrient or any other factor acts, as well as when their genes and genetic networks revealed. In eukaryotic genomes only a small fraction of the DNA codes for proteins, but the non-protein coding DNA harbors important genetic elements directing the development and the physiology of the organisms, like promoters, enhancers, insulators, and non-coding RNA genes. For years, the Crick's central dogma – DNA is transcribed to mRNA and mRNA is translated to proteins – was believed (Crick, 1970). While DNA encoding proteins represents the 2% of the mammalian DNA, the remaining DNA is non-coding sequences (the “junk” DNA). More recently, among this 98%, it was discovered that although not transcribed, some DNA sequences harbors basic information to regulate the transcription of protein-coding genes (e.g. promoters, enhancers, insulators, etc) while other regions are actively transcribed in non-coding RNAs that are essential to assist on protein-coding genes translation (e.g. ribosomal RNAs and transfer RNAs). With the advent of the next generation-sequencing (NGS) technologies, the notion of “junk” DNA was rejected and opened a new gate towards the understanding of the complexity of higher multi-cellular organisms that the protein-coding region fails to explain (Lozada-Chávez *et al.* 2011). Nowadays, high-throughput transcriptomic analyses have revealed that eukaryotic genomes transcribe up to 90% of the genomic DNA (The ENCODE Consortium, 2004). The vast majority of this genomic DNA is transcribed as non-coding RNAs (ncRNAs) either as infrastructural ncRNAs, including ribosomal (rRNAs), transfer (tRNAs), small nuclear (snRNAs) and small nucleolar RNAs (snoRNAs); or as regulatory ncRNAs, mainly classified into microRNAs (miRNAs), P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), promoter-associated RNAs (PARs) and enhancer RNAs (eRNAs; Kaikkonen *et al.* 2011).

2. Non-coding RNAs: families, origins and functions.

Over the last two decades, several evidences were obtained supporting the key role of those regulatory ncRNAs in a diverse set of biological functions in multicellular organism. Although the most of the knowledge came from studies in mammalian species, the widespread and cost-effective use of NGS technologies allowed the identification of these ncRNAs as well as the protein-coding genes involved in their biogenesis and protein-interactions in several taxonomic groups including viruses, bacteria, plants, Cnidaria, Platyhelminthes, insects and non-mammalian vertebrates (fish, birds and reptiles; Rosani *et al.* 2016). Although only few functional studies have been already performed to demonstrate powerful regulatory action of ncRNAs, the appearance of all these ncRNAs along evolution, some of them with regions partially conserved between different taxons, is already a strong evidence of the important role that each type of ncRNAs might have. The most well-known actions of ncRNAs are dealing with the regulation of gene expression, translation and transposon activity. The most studied ncRNAs due to the regulatory action on eukaryotic genes and other genetic elements are the small ncRNAs (sncRNAs): siRNAs, miRNAs and piRNAs (Ghildiyal and Zamore, 2009; Kim *et al.* 2009; Malone and Hannon, 2009), being the last ones the less studied sncRNAs. While siRNAs and miRNAs take part to the same silencing machinery, piRNAs are particularly involved on the silencing of germ-line transposons, among other roles. RNA silencing provides highly specific inhibition of gene expression through complementary recognition of RNA targets. The RNA-induced silencing complex (RISC) forms the core of the RNA silencing machinery, and consists of a protein from the Argonaute (AGO) family and a small RNA that acts to guide RISC to its targets (a 'guide' RNA). Once loaded with a sncRNA, AGO proteins inhibit the expression of their targets, either by cleavage using SLICER endonuclease activity, or by attracting additional proteins that can affect translation, RNA stability or chromatin structure. The majority of eukaryotic organisms possess more than one AGO protein, and the functions of individual members of the family are often non-redundant (Siomi *et al.* 2011). On the other hand, long non-coding RNAs (lncRNAs) can participate in physiological and pathological processes through chromosome inactivation and epigenetic modifications, control of mRNA decay and translation, and/or DNA

sequestration of transcription factors. More recently, circular RNAs (circRNAs) and competing endogenous RNAs (ceRNAs) have been discovered to regulate miRNA function at transcriptional/post-transcriptional level. In addition to their mode of action, those ncRNAs also differed on their biogenesis. The features, biogenesis and mechanisms of action of the most commonly studied classes of regulatory ncRNAs will be briefly described below.

2.1. *Small interfering RNAs (siRNAs)*

The canonical siRNA is a linear double stranded RNA (dsRNA) perfectly base-paired 21–23 nt in length, containing an mRNA sequence (sense strand) and its complement (antisense active strand; reviewed in Wittrup and Lieberman, 2015). siRNAs are processed by DICER endonuclease (as well as miRNAs) that will silence directly the target when loaded onto RISC. In this sense, siRNAs can mediate silencing of mRNA targets (i) at post-transcriptional level; (ii) at transcriptional level by increasing epigenetic marks (e.g. methylation) of heterochromatin, particularly silencing mRNAs of the same locus from which they are derived; and/or (iii) suppressing retrotransposition (reviewed in Castel and Martienssen, 2013). Those functions of siRNAs lead its use in the treatment of human diseases (Wittrup and Lieberman, 2015), as well as a method for gene knockdown system for functional analysis in model species for developmental biology like zebrafish (*Danio rerio*) (Shinya *et al.* 2013), or as a strategy to control viral diseases in aquaculture (Papic *et al.* 2015).

2.2. *MicroRNAs (miRNAs)*

The miRNAs are by far the most extensively studied sncRNAs. They are evolutionarily conserved and small single-stranded molecules (20–24 nt) derived from transcripts (pre-miRNAs) and characterized by forming distinctive hairpin structures (reviewed in Ha and Kim, 2014). Pre-miRNAs are sequentially processed into the mature miRNA by DROSHA and DICER, although a DICER independently processing have been recently demonstrated. Mature miRNAs will finally interact with AGO proteins to form RISC. Then, the miRNAs pair with mRNAs, most favorably to the 3' untranslated region (UTR), although pairing with the 5'UTR or the coding DNA sequence (CDS) has been also demonstrated. In contrast to siRNAs, miRNAs do not perform perfect match with mRNA sequence. Instead,

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a not fully complementary pairing with the 2-8 bases (mer) of the 5'UTR of miRNA seed with the specific mRNA region has been shown in order to regulate the post-transcriptional translation of targeted mRNAs (Yartseva *et al.* 2016). Post-transcriptional regulation of targeted mRNAs can be through deadenylation of the mRNA (followed by its degradation), mRNA cleaving, and/or translation repression at initiation or elongation. Furthermore, in addition to these classical roles, miRNAs have also been shown to regulate gene expression at the promoter of the target mRNA through epigenetic mechanisms. In one or another way, miRNAs could target 30-60% of the human transcribed genes (John *et al.* 2004; Sand *et al.* 2012), having strong implications on cell differentiation and cell death processes, stress responses and diseases. A large set of research works have been performed during the last decade to identify and predict the targeted mRNAs (and biological processes) by the miRNAs in different fish species (see below).

2.3. *P-element–induced wimpy testis (PIWI)–interacting RNAs (piRNAs)*

P-element–induced wimpy testis (PIWI)–interacting RNAs (piRNAs) are known sncRNAs 24-31 nt length, forming complexes with the PIWI proteins from the AGO family, and having a diverse set of functions (reviewed in Siomi *et al.* (2011), Watanabe and Lin (2014), Iwasaki *et al.* (2015), and Sarkar *et al.* (2017), among others). The primary role of piRNAs has been shown to silence transposable elements (TEs) in the germline of animals. TEs are genomic parasites that threaten host genomic integrity as they can move to new sites by insertion or transposition and thereby disrupting genes. In animals, endogenous siRNAs also silence TEs, but the piRNA pathway is at on its forefront. By silencing TEs in the germline, piRNAs prevent harmful mutations from being passed to the next generations. Nevertheless, recent research suggested that piRNAs are playing important roles beyond TE silencing, and being reported their expression in tissues others than gonads (reviewed in Sarkar *et al.* 2017). In brief (reviewed in Siomi *et al.* 2011), piRNAs are originated from single-stranded precursors given rise to antisense piRNAs, which then recognize and target the cleavage of transposons by associated PIWI-proteins. This generates additional sense piRNAs arising from the target transposon sequence. Such process is known as the ‘ping-pong’ cycle, increasing the abundance of piRNAs and transposon silencing. The piRNA mechanism of action is not fully understood but probably involves the arginine methyl-

transferase PRMT5, tudor domain-containing proteins (TDRDs) and the Maelstrom protein (MAEL; Sokolova *et al.* 2011). Interestingly, a recent study showed as piRNA pathway genes rapidly evolved in the teleost fish compared with mammals, likely to adapt to the higher diversity of transposons in the teleost fish species (Yi *et al.* 2014).

2.4. Long non-coding RNAs (lncRNAs)

LncRNAs are ncRNAs longer than 200 nt, although some of them act as a source of shorter RNAs. Categorization of ncRNAs within the lncRNAs is quite ambiguous/heterogeneous regarding its localization. Some lncRNAs are located in the nucleus while others in the cytoplasm. Moreover, some of them can have or not poly A tail (reviewed in Fatica & Bozzoni, 2013). Nevertheless, all of them tend to show a low level of expression. The degree of conservation of lncRNAs along evolution is quite low, being only the 4-11% of lncRNAs retaining some conserved regions between mammals and fish (Basu *et al.* 2013). The lncRNAs have crucial roles in gene expression control during both developmental and differentiation processes (reviewed by Fatica and Bozzoni, 2013). In brief, at the nucleus, lncRNAs guide chromatin modifiers (DNA methyltransferases and histone modifiers, such as the Polycomb repressive complex PRC2 and different histones) to specific genomic loci in order to induce repressive heterochromatin formation and thus, transcriptional repression. Additionally, they also repress gene transcription by acting as decoys sequestering transcription factors, by allosterically modulating regulatory proteins, and/or by altering nuclear domains and long-range three-dimensional chromosomal structures. In this sense, nuclear lncRNAs can act on the same loci (*cis*-) or on a distant one (*trans*-). Cytoplasmic lncRNAs can modulate mRNA translation through a diverse set of modes: by positive or negative regulation with direct complementary pairing with target mRNA, increasing or decreasing mRNA stability, and/or by binding to and sequestering specific miRNAs. A third type of gene expression regulatory mechanism performed by lncRNAs, and the best-characterized one, is through the dosage compensation and genomic imprinting, relying on the formation of silenced chromatin to produce monoallelic expression of specific genes. The extraordinary complexity of transcriptional regulation performed by lncRNAs is further reflected by a single lncRNA working by different mechanisms depending on the cell type.

To date, of the tens of thousands of metazoan lncRNAs discovered by high-throughput transcriptome projects, only a handful of lncRNAs have been functionally characterized. Some of them are XIST, HOTAIR, and MALAT1, the last being evolutionary conserved in fish species (Johnsson *et al.*, 2014). Among them, the H19 gene encoding a 2.3-kb ncRNA is highly expressed during embryogenesis but shut off in most tissues after birth, and known to be involved in genomic imprinting. Maternal undernutrition has been shown to regulate the expression of H19 in a sex-specific manner, being maternal low-protein diet the cause of abnormalities in male but not female mice blastocysts (Kaikkonen *et al.* 2011). In fish species, while other specific lncRNAs have been found to induce developmental defects in zebrafish, such as the lncRNAs *cyrano* and *megamind* (Ulitsky *et al.* 2011), modulation of lncRNAs has been associated with the immune response of Atlantic salmon (*Salmo salar*) to pathogenic infections (Boltaña *et al.* 2016).

2.4.1. Circular RNAs (circRNAs) and Competing endogenous RNAs (ceRNAs)

Among the different classes of lncRNAs, two of them act as sponges for miRNAs. Whereas the linear ceRNAs have a short half-life that allows a rapid control of sponge activity, circRNAs have much greater stability and their turnover can be controlled by the presence of a perfectly matched miRNA target site. circRNAs structurally differ from other lncRNAs in that their 3' and 5' ends are not free but covalently joined at a site flanked by canonical splice signals in contrast to the regular splicing pattern in which a splice donor is joined to a downstream splice acceptor (reviewed in Ebbsen *et al.* 2016). CircRNAs can be derived from exon of protein-coding genes, from intronic, intergenic, UTR regions, ncRNA loci and from locations antisense to known transcripts; and can comprise one or more exons. Interestingly, since some of these multiexonic circRNAs consisted exclusively of exonic sequences, these circRNAs must be subjected to splicing to remove the introns either before or after circularization, and are being exported afterwards from the nucleus to the cytoplasm through an uncharacterized pathway. The role and biological importance of circRNAs is currently uncharacterized and has been a source of debate. Nevertheless, circRNAs have a great stability since no free extremes are found where exonuclease degradation may act, but also the lacking of a 3' poly(A) tail, made them resistant to

deadenylation, decapping and degradation normally observed in mRNAs. Thus, they would be the more efficient sponge for miRNAs as this stability will allow circRNAs to accumulate as well as to maintain the regulatory function for a longer period of time than the ceRNAs. CircRNAs have been predicted and isolated in zebrafish and coelacanth (*Latimeria chalumnae*) (Shen *et al.* 2016) and in large yellow croaker (*Larimichthys crocea*), where GO and KEGG pathway of the genes included in those circRNAs were related with digestive system and metabolism (Xu *et al.* 2017).

2.4.2. Enhancer RNAs (eRNAs)

The eRNAs are another class of lncRNAs. The size of eRNAs range from 0.1 to 9 kb, and show a specific histone methylation signature typical of enhancers (reviewed in Smith and Shilatifard (2014) and Lam *et al.* (2014)). More particularly, eRNAs are produced from DNA regions extremely rich in monomethylation on lysine 4 of histone 3 (H3K4me1) but not so in H3K4 trimethylation (H3K4me3), and are evolutionary conserved. Several evidences indicate that eRNAs function as transcriptional activators, as depleting eRNA led to a gene-specific decrease in mRNA expression. Furthermore, this transcriptional enhancing activity appears to be sequence-specific since substituting eRNA with other open reading frames led to decreased enhancer activity although the transcription start site remained intact (Orom *et al.* 2010). Limited work has been carried out to explore eRNAs in fish species, being only recently and strictly performed on zebrafish eRNAs (Pauli *et al.* 2012; Taminato *et al.* 2016; Trinh *et al.* 2017).

2.5. Overview of the mechanisms of action of ncRNAs

As above-mentioned, each ncRNA can regulate gene expression and translation in a diverse set of ways within each cell. Furthermore, some ncRNAs can also regulate the action of other ncRNAs such as the case of the circRNAs and ceRNAs regarding the action of miRNAs. The different mechanism can be clustered in three types: chromatin remodeling, transcriptional and/or post-transcriptional regulation (reviewed in Kaikkonen *et al.*, 2011).

In one hand, different ncRNAs (lncRNAs and piRNAs) can regulate transcription by recruiting chromatin-remodelling complexes, which in turn mediate epigenetic changes (heritable changes in phenotype and gene expression caused by mechanisms other than

changes/mutations in DNA sequences). A classic example for this model is X-chromosome inactivation, where polycomb group (PcG) proteins binds to ncRNA XIST expressed on the targeted X-chromosome, and initiating and epigenetic silencing by trimethylation of H3K27. PcG also mediates transcriptional repression through interaction with histone deacetylases and exerts long-lasting silencing by CpG (or CG) islands' methylation through interaction with DNA methyltransferase 3 alpha (van der Vlag & Otte, 1999; Viré *et al.* 2006).

On the other hand, ncRNAs can also repress or activate gene transcription. As an example, DHFR gene contains a major and a minor promoter. The lncRNAs generated from the minor promoter bind both the major promoter (triplex formation) and the general transcription factor IIB leading to the dissociation of preinitiation complex, and thus repressing DHFR gene expression (Martianov *et al.* 2007). On the contrary, ncRNAs can also serve as transcriptional coactivators as illustrated by the 3.8 kb polyadenylated EVF2 ncRNA that form a complex with DLX2 and function as a transcriptional activator of DLX5/6 expression in an enhancer-specific manner (Feng *et al.*, 2006).

Finally, ncRNAs can also act at post-transcription level, modulating mRNA splicing, transport, translation and/or degradation. Although piRNAs can act at post-transcriptional level, the most known ncRNAs performing mRNA post-transcriptional regulation are the siRNAs and miRNAs. The difference between siRNAs and miRNAs is the region of action. While siRNAs silence the locus from which they are derived, miRNAs can act in a wider manner affecting genes others than those from its own locus. Some ncRNAs overlaps with (i) the exon-intron boundary, enabling alternative splicing; (ii) the 5' UTR, CDS and/or 3' UTR regions, blocking translation at initiation or elongation; and (iii) the 3' UTR region inducing deadenylation (followed by decapping and degradation of mRNA) or mRNA cleavage (Wilczynska & Bushell, 2014).

3. Non-coding RNAs in circulation.

The complex developmental program encoded on the DNA and its regulation through the action of the different ncRNAs within each cell in an autocrine fashion is further twisted by the paracrine and possibly in an endocrine manner (Viereck *et al.* 2014) when the whole

Fernández-Monzón, I. *et al.*, 2017. Non-coding RNAs: uncovering their potential relevance in fish nutrition. En: Cruz-Suárez, L.E., Rique-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. (Eds), Investigación y Desarrollo en Nutrición Acuicola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 363-389. ISBN 978-607-27-0822-8.

organism is considered. In this sense, the discovery of circulating ncRNAs associated with exosomes and/or lipoproteins opened up the possibility of a holistic and new mediation of gene regulation. A significant and diverse set of ncRNAs have been observed outside the cells, including in different body fluids (reviewed in Allegra *et al.* (2012), Silva & Melo (2015), and Viereck & Thum (2017)). Although the biological consequence of released ncRNAs on distant sites remains to be uncovered, the expression patterns of ncRNAs in body fluids are highly correlated with disease states and other physiological conditions in humans, and being recently associated with sex differentiation in the tongue sole (*Cynoglossus semilaevis*) (Sun *et al.* 2017; see below).

The resistance of ncRNAs to RNases and harsh conditions (e.g. boiling, extreme pH, storage at room temperature, or freezing and thawing cycles), but also its presence in body fluids such as blood, serum/plasma, urine, and breast milk, makes circulating ncRNAs suitable for clinical assessment and monitoring patient's pathophysiological state (Viereck and Thum, 2017). In circulation, ncRNAs transport involve encapsulation into membranous vesicles including exosomes (30–100 nm), microvesicles (100-1000 nm), and apoptotic bodies (500-2000 nm); association to RNA-binding proteins, such as nucleophosmin, AGO2 or lipoprotein complexes like low- and high-density lipoproteins. Since the ncRNA content of extracellular vesicles can differ from that of the parental cell, a specific sorting and packing mechanisms might be favored. The ncRNA content of extracellular vesicles can be taken up by recipient cells, enabling cell-to-cell communication, which is potentially mediated via membrane receptors, vesicle fusion with the membrane of target cells, endocytosis, or remain attached to the plasma membrane activating specific signaling pathways (reviewed in Fritz *et al.* 2016).

Among the ncRNAs found in circulation, miRNAs were the most extensively studied (Allegra *et al.* 2012), and have been almost exclusively characterized in mammalian species. One of the major advantages of miRNAs (as well as other ncRNAs) as mediators of cell-cell communication, is that its presence in body fluids and high stability may represent an infinite resource of non-invasive biomarkers, not only in cancer and other diseases, but also in nutrition. Nevertheless, some challenges still limit its use. In general, the potential lack of specificity of one miRNA for a disease or physiological condition as well as the standardization of the analysis of circulating miRNAs (and other ncRNAs)

regarding the preparation of serum/plasma or the quantification of miRNAs (identification of suitable ‘housekeeping’ serum miRNA/small RNA). Moreover, extra-caution should be paid on the isolation procedure of circulating ncRNAs as for instance, large part of the circulating miRNAs are originated from blood cells (McDonald *et al.* 2011), and thus hemolysis might mask or provide erroneous ncRNAs biomarkers. The potential use of circulating ncRNAs in relevant aquacultured fish species needs further considerations in order to use them as a new biomarkers of physiological condition. In this sense, most of the aquaculture species do not have its genome sequenced, which difficult the prediction of novel ncRNAs but also investigating the biological processes to which there are associated through their targeted sequences (independently of being DNA and/or mRNA sequences). Although the implementation of NGS technologies on aquaculture research and the decreasing prices of such kind of analysis might promote an increasing knowledge on this issue in the nearest future, nowadays an alternative and acceptable approach could be mapping the isolated ncRNAs with close related species where the genome is already known (e.g. Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*), Japanese puffer (*Takifugu rubripes*), tilapia (*Oreochromis niloticus*), and/or zebrafish). Additionally, the development of more powerful and user-friendly bioinformatics tools will also benefit to the advance in this research topic and eventually, it will allow the implementation of therapeutic strategies based on the modulation of specific ncRNAs to improve immune system, allow monosex production and/or solve sexual maturation problems in captivity. However, to achieve these long-term goals functional studies in model species such as zebrafish or medaka (*Oryzas latipes*), where a diverse set of biotechnological tools are available, is still required.

4. Non-coding RNAs in farmed fish species.

The identification and characterization of different ncRNAs in fish species started from an evolutionary point of view, studying miRNAs following a homology approach on the published genome sequences. Posteriorly, this knowledge was expanded with the sequencing of the genome of different fish species. Some research works have been done on the functional characterization of particular ncRNAs in research model fish species

(zebrafish and medaka, mainly). Instead, only few research studies were performed on the characterization of particular ncRNAs in specific traits of farmed fish. In this case, the obvious priority to identify the ncRNAs evolutionary conserved (or not) in aquacultured fish species was translated on their discovery applying *in silico* and/or RNA-sequencing (RNA-Seq) approaches. In this sense, in Table 1, the different studies performed in aquacultured fish species and at distinct developmental stages and/or tissues by high throughput technologies in order to identify ncRNAs are presented.

Research works on ncRNAs were performed in 9 freshwater, 8 marine, 2 catadromous and 1 anadromous farmed fish species, approximately comprising the 51, 32, 6 and 11 % of the studies, respectively. The most abundantly studied fish species are the common carp (*Cyprinus carpio*) (5), the Atlantic salmon (4) and the rainbow trout (4), in line with the fish species from the top-ranking in worldwide production (in Tns and value; FAO, 2016). Surprisingly, an exception to this is the fourth majorly studied fish species, the Atlantic halibut (*Hippoglossus hippoglossus*), with 3 studies, all of them from Dr. Babiak's Lab (University of Nordland, Norway), who is one of the scientific references on ncRNAs research in fish species, mainly regarding the miRNAs.

Among the conducted research, 57 % was on specific tissues (being liver, brain, head kidney and gonads the most frequently studied), 23 % was on different larval developmental stages, and only one work has been conducted in cell lines and another in circulating ncRNAs. Regarding the specific type of ncRNAs studied, the research on miRNAs is the most abundant (comprising almost 83 % of the research studies), being extensively reviewed in Bizuayehu and Babiak (2014), and particularly on those specifically found at the muscle tissue (known as myomiRs; reviewed in Mennigen (2016)). In contrast, only limited, and very recent, research effort has been focus on lncRNAs and piRNAs, representing less than the 12 and 6 %, respectively. Curiously, lncRNAs studies were focus on their association with fish immune response against several pathogens, mostly done by the research group of Dr. Gallardo-Escátare from Chile.

Table 1. Main research works on non-coding RNAs identification by high throughput technologies conducted in aquaculture fish species.

Fish species	Developmental stage/tissue	ncRNAs	Approach	Analysis platform	N° of ncRNAs	Associated biological processes	Reference
<i>Oncorhynchus mykiss</i>	Tissues*	miRNAs	Sanger sequencing		54	Several	Salem <i>et al.</i> 2010
<i>Paralichthys olivaceus</i>	Larvae	miRNAs	Small RNA sequencing / microarray	Solexa	140	Metamorphosis	Fu <i>et al.</i> 2011
<i>Hypophthalmichthys nobilis</i>	Tissues**	miRNAs	Small RNA sequencing		167	Several	Chi <i>et al.</i> 2011
<i>Hypophthalmichthys molitrix</i>	Tissues**	miRNAs	Small RNA sequencing		166	Several	Chi <i>et al.</i> 2011
<i>Lates calcarifer</i>	Tissues***.	miRNAs	Small RNA sequencing		63	Immune system	Xia <i>et al.</i> 2011
<i>Ictalurus punctatus</i>	-	miRNAs	Small RNA sequencing		60	Several	Barozai <i>et al.</i> 2012
<i>Oncorhynchus mykiss</i>	Eggs	miRNAs	Small RNA sequencing	Illumina	496	Egg quality	Ma <i>et al.</i> 2012
<i>Cyprinus carpio</i>	Larvae	miRNAs	Small RNA sequencing		113	Several	Zhu <i>et al.</i> 2012
<i>Hippoglossus hippoglossus</i>	Brain and gonads	miRNAs	Small RNA sequencing	SOLiD	150-168	Sexual differentiation	Bizuayehu <i>et al.</i> 2012a
<i>Hippoglossus hippoglossus</i>	Larvae	miRNAs	Small RNA sequencing	SOLiD	201	Several	Bizuayehu <i>et al.</i> 2012b
<i>Cyprinus carpio</i>	Muscle	miRNAs	Small RNA sequencing	Solexa	195	Muscle	Yan <i>et al.</i> 2012
<i>Ictalurus punctatus</i>	Tissues^	miRNAs	Small RNA sequencing	Solexa	282	Several	Xu <i>et al.</i> 2013
<i>Salmo salar</i>	Juveniles	miRNAs	Small RNA sequencing	Illumina HiSeq 2000	888	Several	Bekaert <i>et al.</i> , 2013

<i>Salmo salar</i>	Tissues^^	miRNAs	Small RNA sequencing	Illumina	193	Several	Andreassen <i>et al.</i> 2013
<i>Hippoglossus hippoglossus</i>	Ovaries	miRNAs	Small RNA sequencing	Roche 454	43	Reproduction	Bizuayehu <i>et al.</i> 2013
<i>Cyprinus carpio</i>	Skin	miRNAs	Small RNA sequencing	Solexa	73	Pigmentation	Yan <i>et al.</i> 2013
<i>Solea senegalensis</i>	Larvae	miRNAs	Small RNA sequencing	SOLiD	320	Thermal plasticity	Campos <i>et al.</i> 2014
<i>Megalobrama amblycephala</i>	Liver	miRNAs	Small RNA sequencing	Illumina HiSeq2000	202	Nutrition	Zhang <i>et al.</i> 2014a
<i>Gadus morhua</i>	Larvae	miRNAs	Small RNA sequencing	SOLiD	389	Thermal plasticity	Bizuayehu <i>et al.</i> 2015
<i>Cyprinus carpio</i>	Epithelial cell line	miRNAs	Small RNA sequencing	Solexa	187	Immune response	Wu <i>et al.</i> 2015
<i>Dicentrarchus labrax</i>	Larvae	miRNAs	Small RNA sequencing	Illumina HiSeq2000	1,928	Several	Kaitetzidou <i>et al.</i> 2015
<i>Anguilla marmorata</i>	Gills	miRNAs	Small RNA sequencing	Illumina Hiseq2500	647	Osmoregulation	Wang <i>et al.</i> 2015
<i>Takifugu rubripes</i>	Tissues#	miRNAs	Small RNA sequencing	SOLiD	1420	Several	Wongwarangkana <i>et al.</i> 2015
<i>Gadus morhua</i>	Larvae	miRNAs	Small RNA sequencing	SOLiD	348	Several	Bizuayehu <i>et al.</i> 2016
<i>Salmo salar</i>	Tissues###	lncRNAs	Small RNA sequencing	Illumina MiSeq	5,636	Immune system	Boltaña <i>et al.</i> 2016
<i>Larimichthys crocea</i>	Whole organism	miRNAs	Genome prediction	-	199	Several	Huang <i>et al.</i> 2016
<i>Oncorhynchus mykiss</i>	Whole organism	lncRNAs	Small RNA sequencing	Illumina HiSeq2000	31,195	Immune system	Paneru <i>et al.</i> 2016
<i>Salmo salar</i>	Tissues###	lncRNAs	Small RNA sequencing	Illumina MiSeq	918	Immune system	Valenzuela & Gallardo, 2016
<i>Oreochromis niloticus</i>	Ovary and testis	piRNAs	Small RNA sequencing	Illumina Hiseq2000	862,289	Gonad development	Zhou <i>et al.</i> 2016
<i>Clarias batrachus</i>	Whole organism	miRNAs	Genome prediction	Illumina HiSeq	210	Several	Agarwal <i>et al.</i> 2017

<i>Colossoma macropomum</i>	Liver and skin	miRNAs	Small RNA sequencing	Illumina HiSeq2000	279	Several	Gomes <i>et al.</i> 2017
<i>Oncorhynchus mykiss</i>	Intestine	lncRNAs	Small RNA sequencing	Illumina MiSeq	9927	Several	Nuñez-Acuña <i>et al.</i> 2017
<i>Oreochromis niloticus</i>	Head kidney	miRNAs	Small RNA sequencing	-	1900	Immune system	Qiang <i>et al.</i> 2017
<i>Cynoglossus semilaevis</i>	Exosomes	miRNAs	Small RNA sequencing	Illumina HiSeq2500	723	Sexual differentiation	Sun <i>et al.</i> 2017
<i>Cyprinus carpio</i>	Ovaries	piRNAs	Small RNA sequencing	Solexa	8765	Several	Wang <i>et al.</i> 2017

*muscle, heart, brain, kidney, liver, spleen, intestine, gill, and skin; ** heart, liver, brain, spleen and kidney; *** Spleen, kidney and liver; ^liver, gill, head kidney, spleen, heart, brain, muscle, stomach, intestines and skin; ^^ liver, spleen, kidney, head kidney, heart, brain, gills, white muscle and intestine; # fast and slow muscles, heart, eye, brain, intestine, liver, ovaries, and testis; ## gills, head kidney, and liver; ### brain, spleen, and head kidney.

The approach followed in all these studies clearly reflected how recent this research topic is. Only one study used individual Sanger sequencing technology, two were based on the prediction through genomic inference, while the remaining 90 % was performed using high throughput sequencing technologies such as Solexa, SOLiD, Roche 454 and Illumina (HiSeq2000, HiSeq2500 and MiSeq) platforms. The output obtained (number of identified ncRNAs) was really variable, depending on the tissue, organ and developmental stage, the type of ncRNA studied and/or the used sequencing platform. The number of ncRNAs identified normally ranges from 43 to 31,195, with only one exception: the identification of 862,289 piRNAs by Zhou et al. (2016) from the gonads of tilapia, tissues known to abundantly express this kind of sncRNAs. Finally, the expression of all those ncRNAs was associated with different biological processes: flatfish metamorphosis, thermal plasticity, immune system, sexual differentiation, gonad development and reproduction, pigmentation, myogenesis, osmoregulation and/or nutrition (extensively reviewed in the case of miRNAs in Bizuayehu and Babiak (2014)). In this sense, as abovementioned, since different ncRNAs are able to regulate genes at transcriptional and post-transcriptional level and there is an intricately genomic program regulating nutrient absorption (at intestine), metabolism (at liver) and transport; a clear role of those ncRNAs on nutrition might be expected.

5. Non-coding RNAs and nutrition

Despite of the abovementioned limited screening of ncRNAs involved in fish nutrition and metabolism, researchers have taking advantage of the wider and deeper knowledge on this issue from mammalian species. In this sense, how maternal nutrition can alter and shape offspring metabolic profile and/or organ function through the alterations in sncRNAs, such as miRNAs, piRNAs, and tRNAs, has been an intense area of research (reviewed in Loche and Ozane (2016) regarding the particular case of cardiovascular diseases). Furthermore, several evidences suggested that not only the endogenously produced ncRNAs might regulate metabolism, but also those obtained from dietary sources (plant foods and cow milk) might affect it by altering the expression of endogenous miRNA genes in mammalian species (recently reviewed in Cui et al.,

2017). Encapsulation of miRNAs in exosomes and exosome-like particles confers protection against RNA degradation and creates a pathway for intestinal and vascular endothelial transport by endocytosis, as well as its delivery to peripheral tissues (see above, section 3: Non-coding RNAs in circulation). Thus, food components and dietary preferences may modulate serum miRNA profiles that might finally influence particular biological processes (Cui *et al.* 2017). Taking into account the relevance of human metabolic disorders nowadays in our society, novel pipelines and databases have been developed, including a dietary miRNAs database reporting miRNAs in 15 dietary resources (Cui *et al.*, 2017). These databases might be examples of future needs to study the diverse types of ncRNAs provided to fish from the diverse source of nutrients in aquafeeds, and mainly considering the nowadays driving force in aquaculture sustainability, the replacement of fish oil and meal by different alternative sources (mainly from vegetable origin). Furthermore, and interestingly, Liang *et al.* (2015) demonstrated in mice the efficacy of lncRNA expression profiles in discriminating the types of microbes in the gut (germ-free, conventional and/or gnotobiotic). Since the gut microbiota is known to have pivotal effects on host physiology, metabolism, nutrition and immunity, such work provided an initial resource of gut microbe-associated lncRNAs for the identification of lncRNA biomarkers in host-microbes interactions. Thus, the question for fish farmers and feed developers could be if this might be also the case of farmed fish.

In one hand, several miRNAs have been associated with energy metabolism in fish species though comparative analysis between mammalian and teleost species, despite the limited work conducted with high throughput technologies (RNA-Seq; see Table 1). For a full list of potential miRNAs involved on the regulation of fish metabolisms, readers are highly recommended the recent review of Mennigen (2016). Some relevant examples of different miRNAs and the related metabolic pathways are as follows. While miRNA-33 in lipid metabolism and miRNA-8163 in iron metabolism have been associated due to the presence of those miRNAs on an intron from SREBP and transferrin genes, respectively; other miRNAs were functionally demonstrated to be linked with metabolism. In this sense, inhibition of liver-specific miRNA-122 was shown to decrease postprandial serum triglyceride concentration and conversely cause postprandial hyperglycemia in rainbow trout (Mennigen *et al.* 2014). Also, inhibition of miRNA-17 in rabbit fish (*Siganus canaliculatus*) regulate, at least partially,

docosahexaenoic acid (DHA) biosynthesis from docosapentaenoic acid (DPA) via fatty acid desaturase 2 (FAD2) mediated Δ^4 desaturation (Zhang *et al.* 2014b). Furthermore, and although poorly understood, some exogenous metabolic stimuli have been found to regulate metabolic miRNAs. In fact, several miRNAs have been reported to be regulated under post-prandial and fasting conditions, as well as by the amount of macronutrients (such as lipids) included in the diets. In this sense, rainbow trout miRNA-122, at the same time regulates lipid homeostasis it was found to be post-prandial regulated (Mennigen *et al.* 2012); increasing abundance of let-7d and miRNA-140-5p in zebrafish was reported when fish were under fasting conditions (Craig *et al.* 2014); and the levels of 12 miRNAs were altered in the liver of blunt snout bream (*Megalobrama amblycephala*) when fed with high-fat diet during a prolonged period, and six lipid metabolism-related genes (*fetuin-B*, *Cyp7a1*, *NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 2*, *3-oxoacid CoA transferase 1b*, *stearoyl-CoA desaturase*, and *fatty-acid synthase*) were bioinformatically predicted to be targeted by those differentially expressed miRNAs (Zhang *et al.* 2014a).

Recently, an analysis of the microRNAs expressed at first feeding larvae from a marine fish species (Atlantic cod, *Gadus morhua*) have provided new insights on how these ncRNAs might mediate nutritional effects on growth (Bizuayehu *et al.* 2016). The miRNAs expressed in larvae fed the golden standard diet for marine fish species (zooplankton, mostly copepods), and showing a better growth and development performance, were compared to the ones found in larvae fed the most commonly used live preys in aquaculture: enriched rotifers and *Artemia*. Eight different miRNAs (miR-9, miR-19a, miR-130b, miR-146, miR-181a, miR-192, miR-206 and miR-11240) were found differentially expressed between the two feeding groups in at least one developmental stage of the six compared, and predicted targets of these miRNAs were associated with metabolic, phototransduction and signaling pathways. Furthermore, since miRNAs have also been shown to regulate gene expression through epigenetic mechanisms at the promoter of the target mRNA, in addition to the post-transcriptional regulation (John *et al.* 2004; Sand *et al.* 2012), these results suggest how first feeding might affect fish growth and development later on, at outgrowth phases, through nutritional programming. Moreover, since strong evidences has been provided that effective amounts of exogenous miRNAs (from cow milk) can be absorbed in humans, physiologic concentrations of exogenous miRNAs affect human gene expression *in vivo*

and *in vitro* (reviewed in Cui *et al.* (2017)); effects on growth and development as well as the modulation of miRNAs in Atlantic cod larvae fed golden standard or regular aquaculture diets could be due to those exogenous miRNAs provided by live preys, which miRNAs were also characterized (Bizuyehu *et al.* 2016).

Finally, and similarly to the associated lncRNAs with mice gut microbiota (Liang *et al.* 2015), Nuñez-Acuña *et al.* (2017) demonstrated how the expression profiles of intestinal lncRNAs in rainbow trout differed when fed for 30 days with functional diets based on pre- and probiotics. Thus, ncRNAs modulation through diets and their role on fish metabolisms seems to be evolutionary conserved, open new research challenges to explore the identification, characterization of ncRNAs in farmed fish, as well as its use as reliable and non-invasive (in the case of circulating ncRNAs) biomarkers of fish nutrition, among other relevant biological processes (e.g. resistance to pathogens, sexual differentiation and reproductive performance).

6. Future perspectives and conclusions.

Considering the several evidences of the ncRNAs playing regulatory roles in fish development and in response to different environmental stimuli (*e.g.* rearing temperature, pathogenic infection, water osmolarity and nutritional composition, among others), as well as other factors like sex and genetic background, an interesting goal in fish farming would be to identify and functionally characterize the full spectrum of ncRNAs with respect to normal *versus* abnormal physiological conditions in most importantly produced species. The development of even further powerful and versatile sequencing technologies, and the decreasing price per sequenced sample would clearly benefit the progress on this research topic. In this sense, RNA-Seq has already been applied to a wide range of issues in fish species (reviewed in Li and Li, 2014) and compared to microarray technology, RNA-seq provides nearly unlimited possibilities in modern bioanalysis since it is not limited towards the amount of RNA, the quantification of transcript levels and the previous knowledge on sequences to be detected and/or quantified. RNA-seq analyses, not only allows the expression level of mRNA, but also the detection and quantification of splice variants, and ncRNAs on a genome-wide scale. The recent and rapid advance on NGS technologies has already made DNA sequencing broadly available, allowing the sequencing of important farmed

fish species, like Atlantic salmon, rainbow trout or turbot (*Scophthalmus maximus*) (Berthelot *et al.* 2014; Lien *et al.* 2016; Figueras *et al.* 2016). Thus, the number of genome sequenced fish species is expected to increase continuously in the coming years. Nevertheless, even in species in which the full genome sequence is still not known, ncRNAs can be identified from the read output from RNA-Seq performing a comparative analysis with the known sequences from other species deposited in relevant ncRNA databases such miRBase (<http://www.mirbase.org/>; Kozomara and Griffiths-Jones, 2014), piRBase (<http://regulatoryrna.org/database/piRNA/>; Zhang *et al.* 2014c), and/or lncRNAdb (<http://www.lncrnadb.org/>; Quek *et al.* 2015) among others. Comparative analysis is a powerful approach to extract functional or evolutionary information from biological sequences (reviewed in O'Brien and Fraser, 2005); however, the low conservation between miRNA-target relationships, especially between teleost fish and mammalian model species, as well as the presence of species specific miRNAs, limit this information gained from this approach in both directions: ncRNAs identification in the species of interest and reliable prediction of the targeted mRNAs or DNA sequences as the conservation of DNA sequences in non-coding regions (*e.g.* gene promoters) is not so high, even when close-related mammalian species are compared (Chiba *et al.*, 2008). Another issue that require attention in such kind of analysis for target mRNA prediction is the existence of different algorithms to rate how the ncRNA-mRNA interaction is most likely to occur. An enormous set of diverse software have been developed in this regard (please, check them at OMICtools; <https://omictools.com/>; Henry *et al.* 2014), being the most used ones miRanda, TargetScan, PITA and/or RNAhybrid for instance (Riffo-Campos *et al.* 2016).

When quantitative studies are in mind to discover new ncRNAs associated with a particular physiological condition, among other factors, correct normalization procedures are required for getting accurate and reliable results. In this sense, although standard and commercial protocols on isolation, treatment and sequencing procedures are available to decrease intersampling variability, normalization by the addition of known amounts of spike-in ncRNAs or by total reads are the most current and accepted procedures in bioinformatic preprocessing analysis (Tam *et al.* 2015). Particularly problematic is the normalization, standardization and quantification of the analysis of ncRNAs in circulation. Avoiding contamination in plasma preparations and finding a reliable 'housekeeping' ncRNA in the serum are the major goals to be pursued. In any

case, an additional and final confirmation of the results obtained from RNA-Seq analysis should be performed by RT-qPCR. Researchers have mainly three options to perform an accurate quantification of sncRNAs: stem-loop, locked nucleic acid (LNA) or linear conventional primers; being stem-loop technology the most sensitive and accurate while the LNA the most specific (discriminate between isomiRs; Nolan *et al.* 2013).

Finally, one of the most important investigations to be carried out in the following years will undoubtedly be the development of functional studies. In this sense, experiments *in vivo* (using model species like zebrafish or medaka) and/or *in vitro* where the knocking down or out of the ncRNAs, and/or the particular inhibition, mimicking or overexpression of miRNAs using synthetically produced miRNAs, will be needed to validate the predicted association of these ncRNAs with a particular physiological condition previously explored by RNA-Seq. In this sense, predicted DNA methylation, chromatin-remodeling and/or transcriptional/post-transcriptional mRNA regulation can be empirically demonstrated.

Concluding, new insights on how genes are regulated by ncRNAs at different levels and ways have been recently gained applying NGS technologies in farmed fish species. This basic knowledge open the doors to new strategies to solve old aquaculture problems such as increasing resistance to pathogenic agents, such the use of siRNAs e application to control viral diseases in aquaculture (Papic *et al.* 2015), early nutritional programming by exogenous miRNAs (Cui *et al.* 2017); and/or as reliable biomarkers of fish immunocompetence in the case of lncRNAs (Boltaña *et al.* 2016; Nuñez-Acuña *et al.* 2017) or miRNAs for metabolic and sexual differentiation conditions (miRNAs; Mennigen, 2016; Sun *et al.* 2017). Furthermore, although the biological consequence of circulating ncRNAs on distant sites remains to be uncover, its characterization in body fluids like plasma might be an interesting approach to allow a continuous and less-invasive monitoring method of fish condition in the nearest future.

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Enfoques Transcriptómicos en el Jurel *Seriola rivoliana*

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Resumen

Las investigaciones con las especies del género *Seriola* van en aumento por la importancia económica que representan para varias regiones del planeta. Mediante las herramientas genómicas, el grupo de Fisiología y Genómica Funcional del CIBNOR en colaboración con otras instituciones, están desarrollando diferentes investigaciones que permitirán conocer la biología de la especie y así optimizar la producción de juveniles. Los enfoques planteados en este trabajo comprenden el estudio de la ontogenia de diversos genes que permiten conocer la evolución de algunos componentes del sistema inmune, así como de factores de crecimiento, proliferación y diferenciación celular, en esquemas básicos de alimentación y en presencia de levaduras como probióticos. Tras estos experimentos, el transcriptoma obtenido representa el primer registro bioinformático en extenso para esta especie y permitirá realizar estudios de biología comparativa, expresión de genes seleccionados bajo contextos experimentales diversos (nutrición, metabolismo, inmunología), y se constituye como una base para la exploración de marcadores genético-poblacionales (SNPs, microsatélites). Por otro lado, se presentan datos preliminares del uso de fitobióticos solos o combinados con probióticos, la presencia de toxinas marinas y su efecto en la expresión de genes relacionados con el sistema inmune y al desarrollo de larvas de jurel *Seriola rivoliana*.

Palabras clave: *Seriola rivoliana*; genómica funcional; nutrición

Herramientas genómicas para el estudio de *S. rivoliana*: RNA-Seq y qPCR

Los peces del género *Seriola* han despertado el interés por la iniciativa privada en diversas partes del mundo, siendo las especies *Seriola lalandi*, *S. dumerili*, *S. quinqueradiata* y *S. rivoliana* las más estudiadas. En México se han establecido diversas empresas en la región noroeste, con la finalidad de cultivar *S. rivoliana*, principalmente para engorda, ya que la producción de juveniles es limitada y aún presenta varios cuellos de botella. Sin embargo, hay pocos estudios sobre la biología básica de esta especie que limita la superación de brechas tecnológicas. Con la implementación de nuevas técnicas de secuenciación masiva de genomas y de transcriptomas, se ha acelerado el conocimiento básico, que nos permite entender aspectos más específicos asociados al desarrollo, metabolismo, crecimiento, nutrición, inmunología y sobre todo de su genética y genómica funcional.

Con el uso de técnicas de genómica funcional como microarreglos, qPCR y RNA-Seq, se han abordado diversos aspectos que nos permiten estudiar efectos sobre numerosos genes relacionados a la inmunología, la digestión, el crecimiento, la diferenciación y proliferación celular y el metabolismo, por ejemplo. Los trabajos de genómica funcional desarrollados por nuestro grupo de trabajo, se han enfocado en *S. rivoliana*, especie cultivada por la empresa Kampachi Farms Co., y que está establecida en el Centro de Investigaciones Biológicas del Noroeste (CIBNOR) mediante un convenio de colaboración.

Los estudios de genómica llevados a cabo por el grupo de Fisiología y Genómica Funcional del CIBNOR, se han direccionado desde diferentes puntos de vista, abordando la nutrición, desarrollo ontogénico, inmunología, metabolismo y salud, para entender cómo los factores bióticos y abióticos del cultivo afectan diversos procesos biológicos que nos permitan optimizar las condiciones de cultivo e incrementar la productividad y calidad desde las primeras fases de su desarrollo.

Información genómica primaria: transcriptoma *de novo*

Para lograr el desarrollo práctico de la investigación a nivel molecular de los procesos fisiológicos, consideramos que la plataforma de inicio, podría ser el uso de la información primaria del genoma traducido, es decir, a partir de la secuenciación de los transcritos.

Las estrategias tradicionales para la obtención de transcritos se basaban en la secuenciación dideoxi-terminal (Sanger) de fragmentos parciales de transcritos denominados ESTs (del inglés *expressed sequence tags*) a partir de la generación de bibliotecas de ADN complementario (ADNc) obtenidas por clonación de fragmentos. Las tecnologías recientes de secuenciación, han permitido facilitar esta tarea, al obtener de forma directa (denominado secuenciación *shotgun*) la secuencia de millones de fragmentos de ADNc para que a través de procesos computacionales de ensambles de secuencias parciales, se reconstruyan los transcritos completos y/o parciales expresados en una muestra biológica. Recientemente, hemos aplicado esta estrategia de obtención del transcriptoma *de novo* en larvas de jurel *S. rivoliana* obtenidas en un cultivo larvario, empleando muestras a los 15 y 30 dpe. Las larvas en ayuno de 12 hrs fueron homogenizadas en TRIzol y el ARN total fue aislado y su integridad fue verificada. A partir del ARN total se generaron librerías de ADNc (TruSeq RNA library kit; Illumina), y las bibliotecas fueron ecualizadas en concentración y secuenciadas en pool en la plataforma NextSeq500 de Illumina, empleando la química de secuenciación de 2x75 ciclos. Con este procedimiento se obtuvieron millones de lecturas totales.

Las secuencias obtenidas fueron depuradas por criterios estrictos de calidad, y los adaptadores fueron removidos con la herramienta Trimmomatic (Bolger *et al.* 2014). Las secuencias depuradas fueron ensambladas *de novo* mediante el algoritmo de Bruijn implementado en el software Trinity (Grabherr *et al.* 2011). Se generaron traducciones conceptuales con el software Transdecoder (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3875132/>) y los transcritos y sus traducciones conceptuales fueron anotadas mediante similitud con la base de datos SwissProt, pFAM y Gene Ontology (Tabla 1).

Tabla 1. Estadísticas descriptivas obtenidas a partir del ensamble *de novo* de transcriptoma de *Seriola rivoliana*

<i>Transcritos ensamblados</i>	117,997
<i>Genes identificados</i>	81,680
<i>Tamaño N50 de transcritos</i>	2,015 pb
<i>Proteínas identificadas</i>	63,344
<i>Proteínas completas</i>	32,015
<i>Parciales internos</i>	10,557
<i>Parciales 5'</i>	5' – 16,325
<i>Parciales 3'</i>	3' – 4,447
<i>Proteínas con anotación SwissProt (evaluate < 1e-5)</i>	45,690
<i>Proteínas con término ontológico asignado</i>	19,154

El transcriptoma obtenido constituye el primer recurso bioinformático extenso para la especie *S. rivoliana*, que permitirá realizar estudios de biología comparativa, expresión de genes seleccionados bajo contextos experimentales diversos (nutrición, metabolismo, inmunología), y es una base para la exploración de marcadores genético-poblacionales (SNPs, microsatélites). Los datos se encuentran en proceso de incorporación a bases de datos públicas para ser disponibles a la comunidad científica.

Nutrición (Aditivos Funcionales)

Desde el punto de vista nutricional, se han utilizado diversos aditivos funcionales como los probióticos, para incrementar la maduración digestiva en las primeras fases del desarrollo, y en la etapa juvenil de algunas especies. En este sentido, se han utilizado prebióticos como β -glucanos y simbióticos para estimular el crecimiento y modular la respuesta inmune innata y adaptativa en peces serránidos, así como aceites esenciales extraídos del orégano, y medicamentos homeopáticos para incrementar la respuesta inmune y antioxidante de *S. rivoliana*.

a. Probióticos: levaduras

La fase larvaria del cultivo de peces representa un cuello de botella tecnológico para las especies que se deseen cultivar, dada la fragilidad, el desconocimiento de su fisiología y la

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falta de esquemas de alimentación apropiados en cada una de ellas. Las altas tasas de mortalidad constituyen una brecha importante tecnológica a superar no solo para la industria, sino también para la parte científica porque limita llevar a cabo estudios relacionados al desarrollo, fisiología digestiva, respuesta inmune entre otros. Una de las alternativas para aumentar las tasas de supervivencia y mejora en la salud de larvas de peces marinos, es el uso de probióticos, cuya utilización se incrementa cada vez más debido a los efectos benéficos que éstos aportan al hospedero. Los efectos más relevantes de la administración de la levadura *Debaryomyces hansenii* son la estimulación del crecimiento, maduración digestiva, supervivencia y estatus antioxidante en diversas especies de peces marinos (Tovar *et al.* 2002, 2004, 2010; Guzmán-Villanueva *et al.* 2008; Reyes-Becerril *et al.* 2008a, 2008b, 2011, 2012; Angulo *et al.* 2017; Tapia-Paniagua *et al.* 2011).

Teniendo lo anterior como antecedente, se realizó un experimento por 30 días en el que las larvas de *S. rivoliana* recibieron la levadura *D. hansenii* como suplemento en la dieta (Burgoin 2015). Las larvas fueron suplementadas con levadura a partir del día 5 post-eclosión (dpe), mediante bioencapsulación de la levadura utilizando como vector el alimento vivo (*Artemia* sp. y rotífero, *Brachionus rotundiformis*) donde el 50% del enriquecedor comercial fue sustituido por la levadura (Fig. 1). Una vez enriquecido el alimento vivo, este se administró a las larvas de *S. rivoliana*.

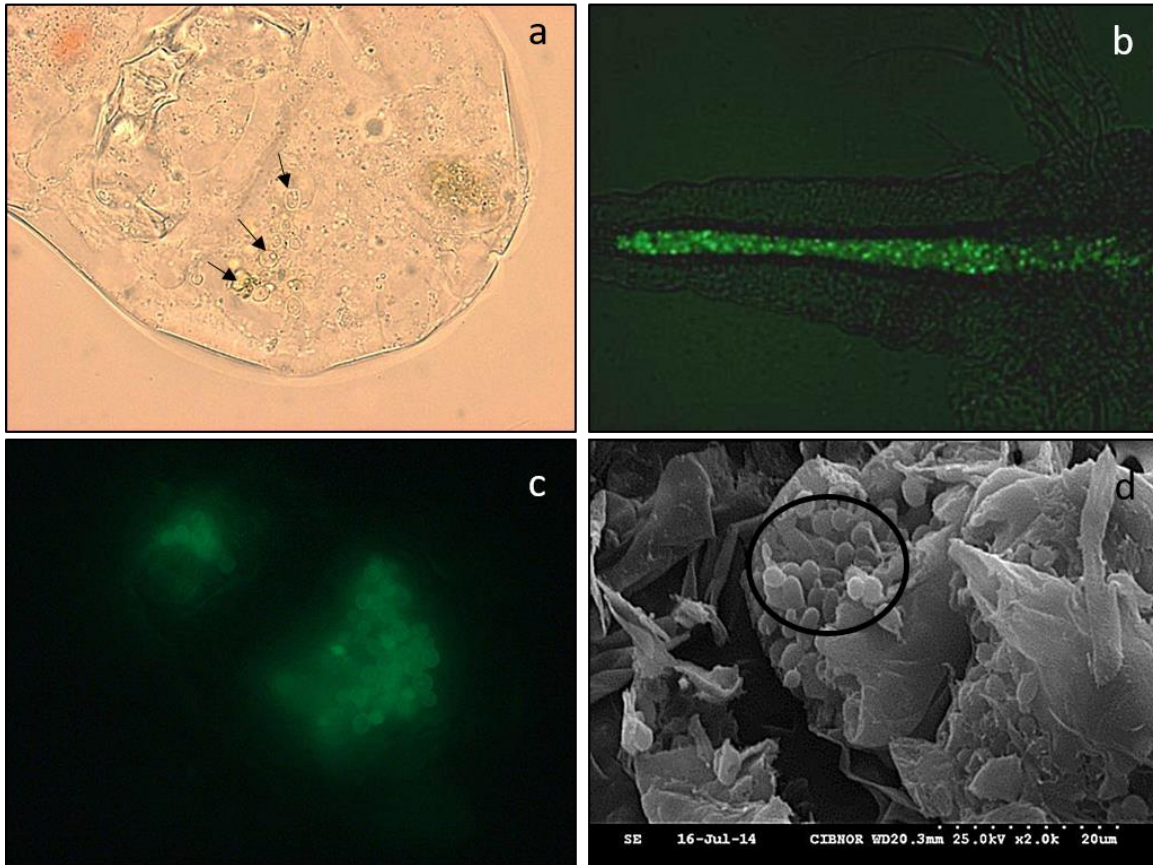


Figura 1. Microscopia de fluorescencia a 40X, donde se aprecian algunas levaduras marcadas con fluoresceína (DTAF) dentro del rotífero y metanauplios de *Artemia* A, B y C. Fig. D microscopia electrónica de barrido a 2000X que muestra el interior del rotífero *B. rotundiformis* con levadura. Flechas y círculo señalan las levaduras en el tracto del rotífero.

El crecimiento y maduración digestiva de larvas de *S. rivoliana* alimentadas con levadura, mostraron un incremento en talla y peso. Por otro lado, para la identificación de los procesos involucrados en la utilización de la levadura como suplemento para larvas de *S. rivoliana*, fueron realizados análisis de expresión génica correspondientes a los procesos de crecimiento, proliferación y diferenciación celular a través de qPCR. Se analizaron 6 genes de interés donde se pudo observar una mayor expresión del *antígeno nuclear de proliferación celular* (PCNA), *proteína morfogénica ósea* (BMP) quien juega un papel clave en el desarrollo óseo induciendo la diferenciación de células mesenquimales en los precursores de osteoblastos; y el *colágeno del tipo 1a1* (COL1a1) encontrado en la mayoría de los tejidos

conectivos, así como en el hueso. Aunque no se pudo observar diferencias estadísticas significativas en el *Factor de crecimiento insulínico tipo 1* (IGF1) a los 30 dpe, se pudo observar una mayor expresión del IGF1 en las larvas suplementadas con levadura al día 15 (datos no mostrados). Para el IGF2 y la *hormona de crecimiento* (GH) no se observaron diferencias significativas entre las larvas del control y las alimentadas con levadura al final del experimento (Fig. 2).

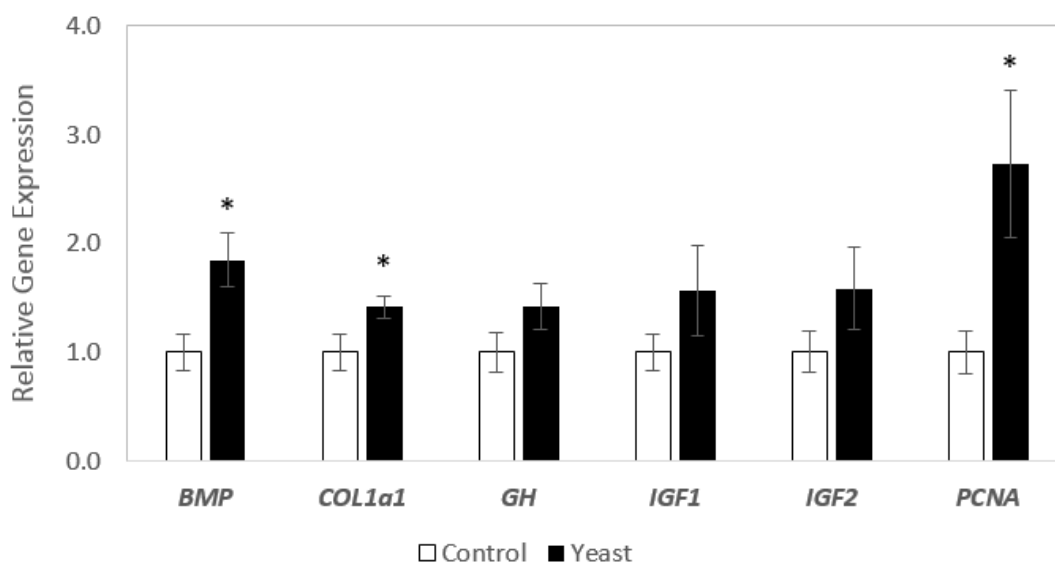


Figura. 2. Expresión relativa de diversos genes involucrados en el crecimiento, inmunología y diferenciación celular de larvas de *S. rivoliana* a los 30 días después de la eclosión en peces alimentados con (Yeast) y sin levadura (Control). La expresión relativa de los genes diana ha sido normalizada al gen de referencia *Factor de elongación 1* (EF1) y de la *Subunidad 18 s ribosomal* (18S). Los datos representan la media \pm D.S. (n=3) de incremento en relación al control (1.0). El asterisco indica diferencias estadísticas significativas entre larvas Control y alimentadas con la levadura (*t*-test, $p < 0.05$).

La expresión de genes clave para el desarrollo, como aquellos involucrados en rutas metabólicas como la de la vitamina D, la síntesis de diversas enzimas digestivas (hidrolasas), metabolismo de polisacáridos, lípidos y actividad ATPasa también fue aumentada (resultados no mostrados). Por otro lado, actualmente se está procediendo al estudio de las rutas

metabólicas estimuladas por la presencia de levaduras, particularmente las involucradas en la proliferación y diferenciación celular, no sólo a nivel morfo-histológico sino también utilizado aproximaciones transcriptómicas de mayor rendimiento como la identificación de los miles/cientos/decenas de genes diferencialmente expresos entre las larvas Control y las alimentadas con la levadura por RNA-Seq.

Para conocer el efecto de los diferentes aditivos funcionales utilizados, es necesario contar con marcadores moleculares de maduración digestiva y respuesta inmune; de tal manera que a lo largo de los primeros 30 días del desarrollo del jurel, se cuantificó la expresión de los genes *Interleukina 1 beta* (IL1B), *Interleukina 10* (IL10), *Gen 88 de respuesta a diferenciación mieloide primaria* (MYD88), y *Fosfatidilinositol sintetasa* (PIS) (Fig. 3). El objetivo de este trabajo fue conocer el patrón de expresión de algunos genes que codifican moléculas relevantes para diferentes respuestas del sistema inmune durante el desarrollo ontogenético. El perfil de expresión se muestra en la Fig. 3.

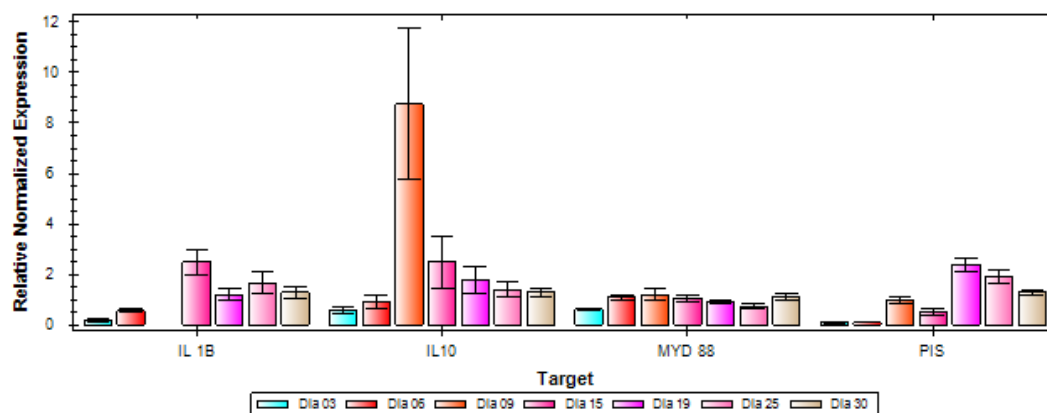


Figura 3. Ontogenia de expresión de algunos genes del sistema inmune como la *Interleukina 1 beta* (IL1B), *Interleukina 10* (IL10), *Gen 88 de respuesta a diferenciación mieloide primaria* (MYD88), y *Fosfatidilinositol sintetasa* (PIS) en larvas de *S. rivoliana* a diferentes días post eclosión. Valores relativos al promedio de la expresión de los genes *Ef1* y *18s* de *S. rivoliana*.

El análisis de qPCR mostró incrementos significativas para IL-1B al día 15, y en el nivel más bajo de expresión se observó el 03 dpe, y ausencia de transcritos para el 09 dpe. Para IL-10 se observó un incremento significativo de la expresión al 09 dpe con respecto al tiempo y a los niveles de los otros genes analizados en este experimento. MyD88 se expresó constantemente y no presentó variaciones significativas en su regulación a través del tiempo. El nivel más alto de expresión para PIS fue el 19 y 25 dpe.

El conocimiento del desarrollo larvario, así como el optimizar la nutrición y reforzar la respuesta inmune, son aspectos que permiten abatir las mortandades de los peces que están siendo considerados como especies emergentes para la industria.

b. Fitobióticos: Experiencias en la combinación de aceite esencial de orégano (*Lippia graveolens*) y probióticos (*Debaryomyces hansenii*)

Recientemente, y aunque en menor escala, los fitobióticos han sido utilizados como inmunoestimulantes, aunque con resultados prometedores (Van Hai *et al.* 2015). Se definen como productos obtenidos de plantas que son añadidos al alimento para incrementar el rendimiento de animales destinados al consumo humano y generalmente incluyen hierbas, especias, aceites esenciales y oleoresinas entre otros (Windisch *et al.* 2008). En los últimos años, se ha estudiado el efecto inmunomodulador de algunos aceites esenciales y de compuestos aromáticos incluidos en ellos, principalmente los terpenos. Entre estos compuestos, el carvacrol es un inmunomodulador y promotor del crecimiento en peces. Por otro lado, sus efectos en el sistema inmune se han estudiado a nivel celular, molecular e histológico en diversos modelos animales (Karkabounas *et al.* 2006; Lee *et al.* 2008; Hotta *et al.* 2010; Choet *et al.* 2012; Lima *et al.* 2013).

La combinación de probióticos y fitobióticos podría constituirse como una estrategia responsable con el medio ambiente en el desarrollo de futuros aditivos funcionales para peces. El trabajo de Hassan y Soltan (2016), es el primer estudio que explora la posibilidad de incluir aceites esenciales de plantas medicinales y bacterias probióticas al mismo tiempo, obteniendo buenos resultados en el crecimiento y en algunos parámetros relacionados con la salud de los peces. Recientemente, Hernández-Contreras *et al.*, (com. personal) incorporaron

aceite esencial de orégano y la levadura *D. hansenii*, por separado y en combinación, en piensos para juveniles de *S. rivoliana*. En estos peces se analizó los efectos sobre el crecimiento, el sistema inmune innato, el estatus oxidativo y el crecimiento luego de seis semanas de alimentación con 4 dietas: control (sin aditivos), con levadura viva (1.1% de inclusión), con 500 mg kg⁻¹ de aceite esencial de orégano (OEO), y levadura + orégano. Como resultado inicial, se obtuvo que los peces aceptaron adecuadamente todas las dietas experimentales con factores de crecimiento y conversión alimenticia excepcionales con respecto a otras especies y similar al reportado por Kissinger *et al.* (2016). Los resultados obtenidos en parámetros sanguíneos, mucus y expresión de genes del sistema inmune mostraron un efecto superior con el uso de los aditivos por separado, y más potente en el caso de la levadura. Sin embargo, la combinación de los tratamientos tuvo un efecto negativo. Por otro lado, el mayor nivel de expresión de MyD88 en riñón cefálico, con un papel central en el sistema inmune, se observó con la dieta que contenía aceite esencial de orégano; el resto de genes se expresaron con un nivel más alto en el grupo alimentado con la dieta con levadura. En los peces alimentados con la combinación de aditivos, los tres genes relacionados con el sistema inmune fueron sub-expresados con respecto a los de la dieta control (Fig. 4).

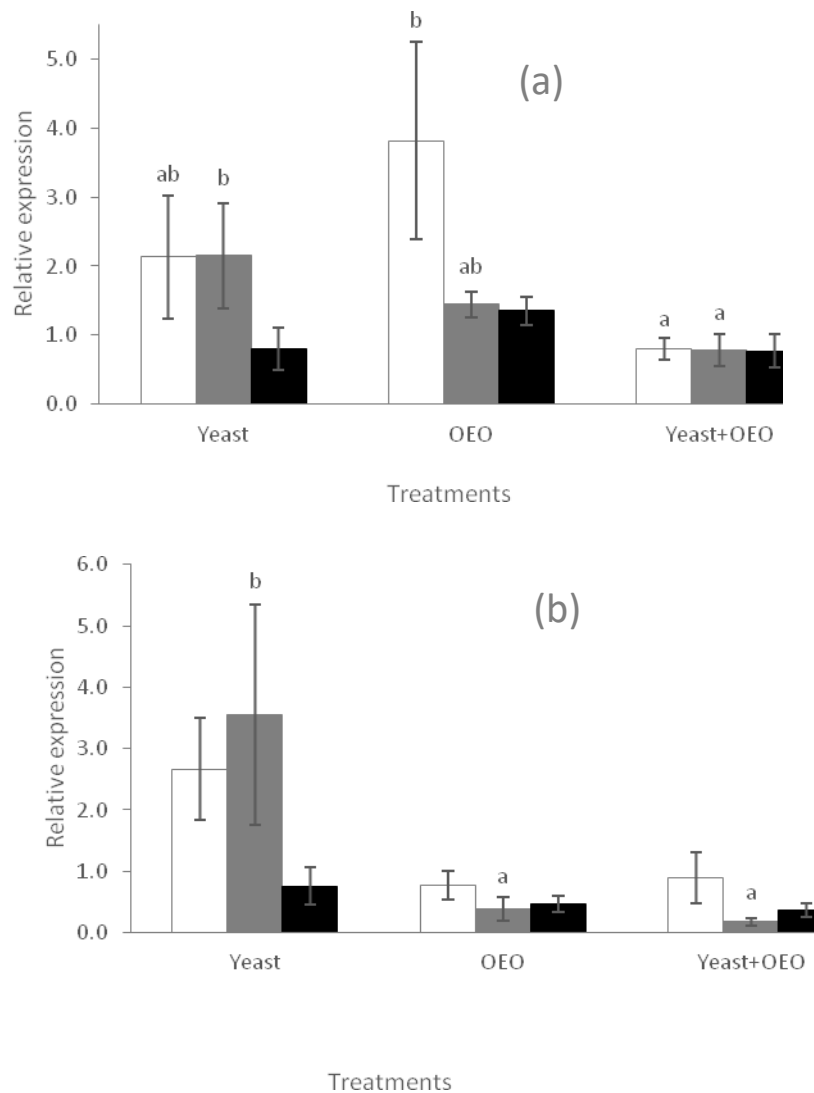


Figura 4. Expresión relativa de los genes *Gen 88 de respuesta a diferenciación mieloide primaria* (MyD88) (blanco), *Tumor necrosis factor* (TNF- α) (gris) y piscidina (negro) en el riñón cefálico (a) y en el bazo (b) de jurel alimentado durante seis semanas con cuatro dietas (control, *D. hansenii* (Yeast), aceite esencial de orégano (OEO) y *D. hansenii* + OEO (Yeast + OEO)). Los datos representan la media \pm D.S. (n=3) de incremento en relación al control (1.0). Letras diferentes expresan diferencias significativas entre grupos (p < 0.05).

Salud

a. Toxicogenómica

Uno de los mayores retos en los estudios de toxicología, es conectar la respuesta de genes con los fenotipos estresantes ambientales (Pavey *et al.* 2012). Para hacer frente a este desafío se requiere integrar enfoques interdisciplinarios que permitan entender los procesos que actúan en los diferentes niveles de organización, desde los ecosistemas hasta los genes. Conocer los efectos de las toxinas en el transcriptoma, es una herramienta comúnmente utilizada hoy, ya que muestra una visión crucial de los procesos biológicos afectados y su mecanismo de acción.

Dada la problemática de la aparición de intoxicaciones producidas por diversas toxinas marinas en organismos de importancia económica y su repercusión en la salud humana por su consumo; y por otro lado, el crecimiento y futuro de la industria de cultivo de peces marinos, es importante efectuar investigaciones para determinar los mecanismos implicados en la toxicidad, así como encontrar métodos de prevención y monitoreo. En el CIBNOR, se ha abordado esta problemática desde varios puntos de vista interdisciplinarios, desde aislamiento, identificación y caracterización de las toxinas producidas por organismos marinos hasta el establecimiento de métodos moleculares de detección, y programas de monitoreo.

El ácido okadaico (AO), entre otras toxinas marinas, es producido por varias especies de dinoflagelados de los géneros *Prorocentrum* y *Dinophysis*, siendo responsable de problemas de salud humana asociados al consumo de algunos mariscos, tales como náusea, vómito, diarrea, dolores abdominales (Aune *et al.* 2012), se ha comprobado que inhiben las proteínas fosfatasa del tipo 1 (PP1) y 2A (PP2A) (Biolojan & Takai, 1988), implicadas en varios procesos intracelulares importantes como la contractibilidad, metabolismo, transcripción y mantenimiento del citoesqueleto (Traoré *et al.* 2003; Ehlers *et al.* 2010). Otro ejemplo es la saxitoxina (STX) y análogos (PSP) son toxinas paralizantes, solubles en agua y que actúan bloqueando los canales de sodio de las neuronas y son producidas por algunos dinoflagelados y algunas cianobacterias en mares tropicales y subtropicales, pero también en agua dulce (Da

Silva *et al.* 2014). Algunos reportes, aunque no concluyentes, indican que existen evidencias de genotoxicidad asociada a eventos de estrés oxidativo, por lo que algunos de los mecanismos de la toxicidad aún son desconocidos en peces y eso representa una oportunidad de estudio importante para la caracterización de su impacto en la industria agroalimentaria relacionada con la pesquería y/o la acuicultura.

Debido a que los peces son componentes de los sistemas acuáticos y que constituyen parte importante de la cadena trófica y alimento para los humanos, actúan como centinelas ambientales en la ecotoxicología. El pez cebra y la tilapia son peces dulceacuícolas que han sido usados como modelo en ecotoxicología (Zhou *et al.* 2005), sin embargo, los eventos de toxicidad más importantes y donde ocasionan pérdidas millonarias, se dan en el medio marino, que constituye el medio donde se generará la futura producción que surtirá la cadena alimenticia humana. Los trabajos que se realizan por el grupo de Fisiología y Genómica Funcional en esta área, han permitido conocer el efecto de la presencia de toxinas marinas sobre el desarrollo embrionario y larvario del jurel *S. rivoliana*, lo cual constituye un factor limitante en la disponibilidad de larvas y juveniles en su ambiente de reclutamiento.

Para conocer el efecto de la presencia del AO y STX durante el desarrollo embrionario y larvario inicial de *S. rivoliana*, se desarrollaron varios experimentos en el CIBNOR para conocer la respuesta transcriptómica en estas etapas. Por un lado se observó que la expresión de la *proteína morfogénica ósea* (BMP) y del *antígeno nuclear de proliferación celular* (PCNA), disminuyen naturalmente de las 8 a las 28 horas del desarrollo embrionario, pero en dosis bajas de ácido okadaico se estimula su expresión (Fig. 5 y 6). En el caso de la BMP, ésta es requerida durante la embriogénesis para la organización del segmento caudal y mesodermo ventral, evitando la formación de dos colas por ejemplo cuando su expresión se ve alterada. La expresión de la PCNA es importante durante las primeras horas del desarrollo por la proliferación celular acelerada y posterior diferenciación en los diferentes tejidos y órganos. Ambos valores de expresión son modificados durante la embriogénesis, pero mayormente significativos en dosis bajas de AO (Fig. 6).

Desde el punto de vista del metabolismo lipídico, la presencia de toxinas marinas como el AO y la STX inducen la expresión de genes como el de la lipasa pancreática, sin embargo, ambas toxinas inhiben el desarrollo embrionario y la eclosión.

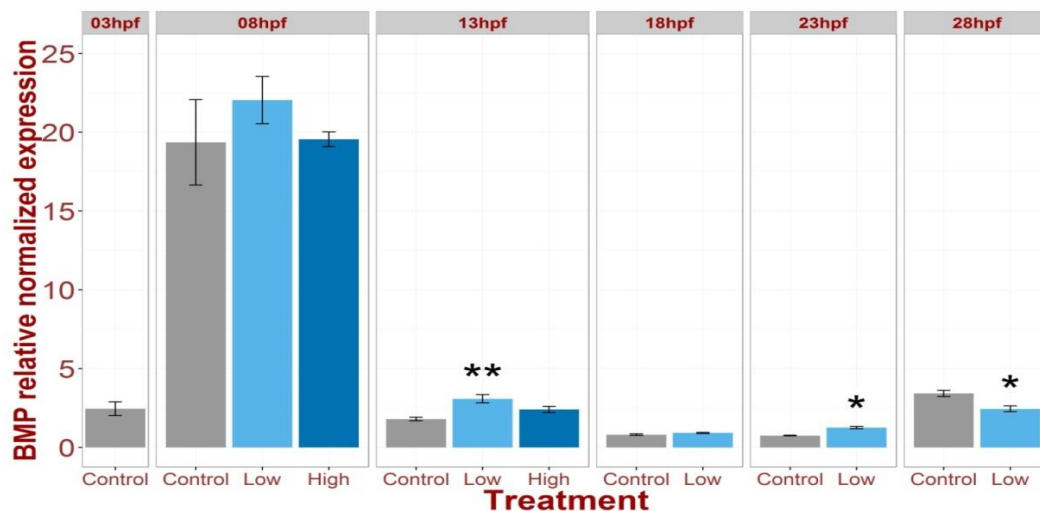


Figure 5: Valores medios de expresión relativa de la *proteína morfogénica ósea* (BMP) en embriones de *S. rivoliana* expuestos al ácido okadaico (AO) durante 28 horas post-fertilización (hpf). Control: huevos cultivados sin AO; Low: huevos cultivados a dosis baja de AO ($120\mu\text{g}\cdot\text{l}^{-1}$ AO eq.), High: huevos cultivados con dosis alta de AO ($175\mu\text{g}\cdot\text{l}^{-1}$ AO eq.). Valores con diferencia significativa contra el control son señalados con “*” y “**” con significancia de $p < 0.05$ y $p < 0.01$, respectivamente. Para la expresión está referida al gen de referencia Efl

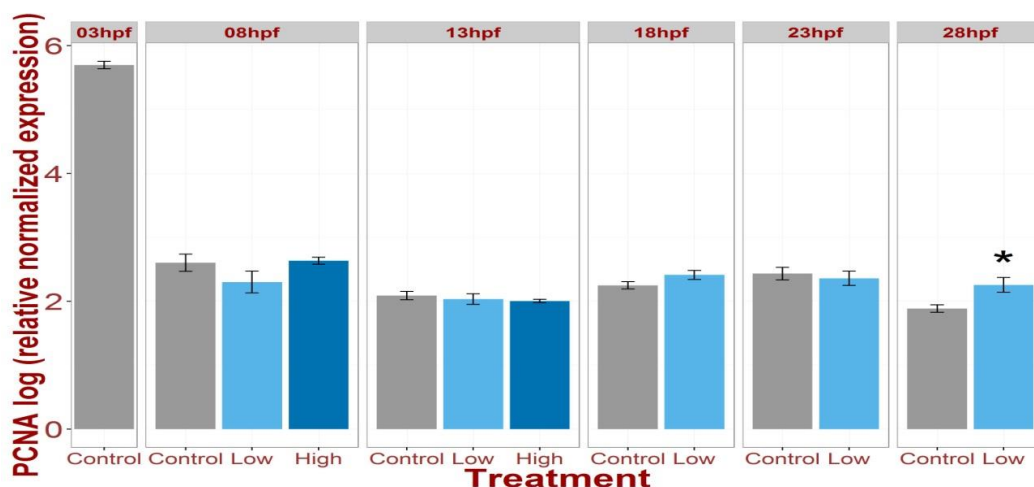


Figure 6: Valores medios de expresión relativa del *anticuerpo nuclear de proliferación celular* (PCNA) en embriones de *S. rivoliana* expuestos al ácido okadaico (AO) durante 28 horas post-fertilización (hpf). Control: huevos cultivados sin AO; Low: dosis baja de AO ($120\mu\text{g}\cdot\text{l}^{-1}$ AO eq.), High: huevos cultivados con dosis alta de AO ($175\mu\text{g}\cdot\text{l}^{-1}$ AO eq.). Valores con diferencia significativa contra el control son señalados con “*” y “**” con significancia de $p < 0.05$ y $p < 0.01$, respectivamente. Para la expresión está referida al gen de referencia Efl.

Perspectivas

Los estudios de nutrigenómica y transcriptómica aportan conocimiento de vanguardia y complementan las técnicas tradicionales, aportando un conocimiento para describir la biología de nuevas especies de interés en acuicultura. Los estudios que actualmente se desarrollan en el CIBNOR con el jurel *Seriola rivoliana*, incluyen la fisiología digestiva bajo diferentes aditivos funcionales, inmuoestimulación temprana con medicamentos homeopáticos y uso de fuentes proteicas alternativas para su engorda y el uso de embriones para describir procesos de toxicología y metabolismo lipídico, son solo algunos de los ejes de investigación que darán como resultado la generación de conocimiento de frontera, con aplicabilidad en el desarrollo, innovación y mejora continua de protocolos de cultivo y tecnologías de producción de esta especie.

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Change in Protein Digestion Capacity During Juvenile Fish Ontogeny: Approach on Spotted Rose Snapper (*Lutjanus guttatus*)

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Abstract

Aquaculture is facing a challenge in order to search new alternative nutritional sources to generate highly digestible and profitable diets for aquaculture species. In addition, the understanding of changes in digestive capacity in fish species with aquaculture potential is of relevance importance, as the capacity of assimilation of different nutrients may change during the juvenile development of the species. Numerous research has been focused on understanding the changes and adaptations of the development and capacities of the digestive system during the early ontogeny of fish, minimizing the importance of possible changes during juvenile ontogeny, as a trigger for the grow-out efficiency increase in fish culture.

Thus, few studies address the digestive changes during juvenile fish ontogeny and their implications in the ability to assimilate different nutritional sources, considering that there should be no changes during this stage, which in general represents the period of grow-out until commercial size, prior to their reproductive stages.

The present work deals with the importance to characterize changes the digestive capacity during grow-out on the spotted rose snapper (*Lutjanus guttatus*). Comparative studies of juvenile sizes of the species (20 to 400 grams) have shown existence of changes in the optimum alkaline protease activity, as well as a diversification and increase in the number of digestive enzymes of the alkaline phase in relation to juvenile ontogeny, resulting in changes of *in vitro* hydrolysis degree and total release of amino acids from different protein sources.

Keywords: Proteases, enzyme characterization, electrophoresis, *in vitro* digestibility, Lutjanidae.

Introduction

Based on their eating habits and digestive morphology, fish are classified as detritivores, herbivores, omnivores or carnivores. Independent of the classification of dietary habits, fish are able to modify their digestive behavior and metabolism in response to changes in dietary sources as well as food availability (Rust, 2002; Pérez-Jiménez *et al.* 2009). Thus, growth and efficiency caused by the ingestion of a food in fish will depend on its physiological and biochemical ability to digest and transform nutrients, however many biotic and abiotic factors can influence the physiological state of the fish and therefore, the processes related to the digestion, absorption and transformation of these nutrients (Furnè *et al.* 2008).

Due to the rapid growth of the aquaculture sector, as well as the emergence of new species with aquaculture potential, there are currently a large number of researches related to development of formulated diets with the objective to search new ingredient sources of low cost and high digestibility. During ingredients searching, it is important to understand the digestive capacity of the species of interest, where the understanding of the number and type of digestive enzymes, their enzymatic activity, as well as the affinity that present to different nutritional sources, will be important for the design of new formulations that tend to generate a sustainable aquaculture industry.

From digestive enzymes, proteases play a key role in digestion, which translates into high growth and survival. The proteases found within the digestive organs of fish are responsible for catalyzing the hydrolysis of peptide bonds (Klomklao, 2008), which includes enzymes such as pepsin, gastricins, trypsins, chymotrypsins, collagenase, elastase, carboxypeptidases and carboxylesterases (Haard, 1994; Simpson, 2000), where trypsin, chymotrypsin and pepsin are considered as the most important digestive enzymes due to their abundance and high proteolytic activity according to studies in different fish species (Castillo-Yáñez *et al.* 2004, 2005, 2006; Klomklao *et al.* 2004, 2007).

In fish, a great effort has been made in understanding the changes in physiological digestive capacity during the early ontogeny of different fish species, which has promoted the development of zootechnics in larval and juvenile production of fish (Kolkovski, 2001; Zambonino-Infante and Cahu, 2001; Lazo *et al.* 2007; Rønnestad *et al.* 2007; Álvarez-

González *et al.* 2008; Galaviz *et al.* 2012; Salze *et al.* 2012; Moguel-Hernández *et al.* 2013).

Digestibility of nutrient or diet depends on its chemical composition, type of ingredients and digestive capacity of the species to breakdown macronutrients to micronutrients to be absorbed (Lemos & Tacon 2011). The major criteria to determine the nutritional value of protein sources seems to be apparent digestibility coefficient (ADC) (Dimes *et al.* 1994) in which, total assimilation (digestion and absorption) of specific nutrients are obtain by feces collection and analyses. By the other side, pH Stat system is a practical tool to conduct *in vitro* measurement using the degree of hydrolysis (DH%) as criteria, providing multiple advantages such as: specific response by using standardized species enzymes, stable conditions, rapid, precise, test different ingredients in small amounts and appropriate for different ingredients sources, include marine-based, animal and plant ingredients (Lemos *et al.* 2009; Yasumaru & Lemos 2014). Actually, great interest and efforts exist for standardization of pH Stat method in fish species (Dimes *et al.* 1994; El-Mowafi *et al.* 2000; Tibbetts *et al.* 2011a, b; Yasumaru & Lemos 2014) and crustaceans (Ezquerria *et al.* 1997; Lemos *et al.* 2000; Lemos *et al.* 2009; Perera *et al.* 2010), because main limitation in pH-Stat assays seems to be the complete knowing of enzymes origin and activities, given that, variations in species, fish size/age and phenotype could generate poor reproducibility over *in vitro* digestion assays (Tibbetts *et al.* 2011a).

Few studies in aquatic organisms aboard enzyme changes or diversification during juvenile or adult ontogeny in a same species. Reports in species such as roach (*Rutilus rutilus* L.), cuban gambusia (*Gambusia punctata*), Japanese eel (*Anguilla japonica*) and tilapia (*Oreochromis niloticus* L.) showed that proteolytic activities and zymogens could differ during juveniles/adults stages in the same species (Chiu & Pan 2002; Kuz'mina 1996; Falcón-Hidalgo *et al.* 2011; Unajak *et al.* 2012).

Some consistent examples about advantages related to the presence of some digestive isoenzymes in aquatic organisms exist, where oyster (*Crassostrea gigas*) presents a genetic polymorphism in two alpha-amylase genes (AMYA and AMYB), that are related to growth (Prudence *et al.* 2006). Spiny lobster (*Panulirus argus*) presents genetic variation in digestive trypsin pattern (three phenotypes; A, B and C), that generate *in vitro* differences in digestion efficiency over different protein sources (Perera *et al.* 2010, 2015).

In this sense, Atlantic salmon (*Salmo salar*) is the most studied fish, where fish possessing a certain trypsin phenotype (TRP-2*92), shows better growth rate and/or feed conversion efficiency, related with protein digestion capacity (Bassompierre *et al.* 1998; Rungruangsak-Torrissen *et al.* 1998; Torrissen *et al.* 1987; Torrissen 1991).

Hence, understanding of digestive physiological aspects that directly affects feed efficiency and growth in the target species is required. The species studied is the spotted rose snapper (*Lutjanus guttatus*), which is part of the Lutjanidae family, consisting of predatory fish with variable feeding habits, where all are carnivores, feeding mainly on fish and benthic crustaceans (Allen 1987; Vázquez *et al.* 2008). In the species, great efforts have already been made in the search for alternative protein sources for the manufacture of feeds in the juvenile stage, in order to reduce dependence on fishmeal, however studies focused on differences in capacity digestion during juvenile ontogeny are lack. Therefore, the objective of the present work was to characterize the digestive proteases in a comparative way during the juvenile ontogeny of the spotter rose snapper and determine the possible differences in protein digestive capacity using *in vitro* techniques.

Material and Methods

Experimental animals

Fish for this study were obtained from the Laboratory of Reproduction and Marine Finfish Hatchery (CIAD), Sinaloa, México, where all juvenile stages were obtained from single spawning batch, conducted as described by Álvarez-Lajonchère *et al.* (2012). After one batch larval culture, all juvenile fish continued under normal culture (nursery step) and fattening process, until were collected in different times from one cycle. According to their wet weight, fish were classified in three groups (all considered in the juvenile stage): early juvenile (EJ; 21.3 ± 2.6 g; 3 months after hatchery, MAH), middle juvenile (MJ; 190 ± 4.4 g; 7 MAH) and late juvenile (LJ; 400 ± 11.5 g; 12 MAH). Fish were adapted to control diet reported by Silva-Carrillo *et al.* (2012). Fish were starved for 24 hours to ensure the emptiness of the gut, euthanized ethically by a single puncture in the head with scalpel and immediately dissected to extract the digestive tract.

Dissection and extract preparation

The digestive tract of each fish was individually divided into five segments: stomach (ST), pyloric caeca (PC), and intestine in three sections (proximal (PI), middle (MI) and distal intestine (DI)). All of the procedures were conducted at temperatures of 0-4 °C. All segments were frozen individually at -64 °C until the assay was conducted. Prior to analysis, segment was diluted in a ratio of 1:10 (wet weight: volume) in a physiological saline solution (NaCl 9g L⁻¹) and ice-cold-homogenized with an Ultra-Turrax homogenizer. Homogenates were centrifuged (8500 × g) at 4 °C for 15 min, and the supernatant was used to perform enzyme activity assays (Matus-de-la-Parra *et al.* 2007).

Enzyme activity assay

The pepsin-like or total acid protease activity was measured by a modified method of Sarath *et al.* (1989), with denatured hemoglobin (2 % pH 2) as substrate. Alkaline protease activity was estimated by method of Walter (1984) using casein as substrate. The trypsin activity was determined by modified method of Erlanger *et al.* (1961), using N α -benzoyl-L-arginine-4-p-nitroanilide hydrochloride (BAPNA 1 mmol L⁻¹) as substrate. The protein content of the supernatant solution was determined by Bradford assay (1976) using bovine serum albumin as the standard. One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 μ g of product released per minute. Tyrosine amount liberated from haemoglobin and casein hydrolysis was determined at 280 nm, while amount of p-nitroaniline liberated from BAPNA was determined at 410 nm.

$$1) \text{ Total activity (Units ml}^{-1}\text{)} = [\Delta\text{abs} \cdot \text{reaction final volume (ml)}] / [\text{MEC} \cdot \text{time (min)} \cdot \text{extract volume (ml)}]$$

$$2) \text{ Specific activity (Units mg prot}^{-1}\text{)} = \text{Total activity} / \text{soluble protein (mg)}$$

Δ abs represents the increase in absorbance, and MEC represents the molar extinction coefficient of tyrosine or p-nitroaniline (0.005 and 0.008 mL/ μ g/cm, respectively).

Characterization of digestive enzymes

Pepsin-like, total alkaline protease and trypsin were characterized by determining the relative activity (%) as a function of pH and temperature. The temperature effect for pepsin-like was measured from 10 to 50 °C; alkaline protease and trypsin were measured

from 10 to 60 °C, with similar assay conditions as previously described. The pH effect on digestive activity was measured at 37 °C, and the buffers were in range from pH 1 to 10 using buffers as previous described by Matus-de-la-Parra *et al.* (2007).

In addition, characterizations of acid and alkaline proteases were performed according to Guerrero-Zárate *et al.* (2014) using specific inhibitors. Pepstatin A (1 mmol L⁻¹) was used as an inhibitor of acid proteases from stomach and alkaline protease activity inhibition in pyloric caeca and intestine sections were performed using the following inhibitors: 250 mmol L⁻¹ soybean trypsin inhibitor (SBT1), 10 mol L⁻¹ N-tosyl-L-phenyl-chloromethyl ketone (TPCK), 100 mmol L⁻¹ phenylmethylsulfonyl fluoride (PMSF), 10 mmol L⁻¹ N_α-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 10 mmol L⁻¹ 1,10-Phenanthroline (Phen) and 250 mmol L⁻¹ Type II-Turkey egg Ovomuroid (Ovo).

Chemical analysis

The moisture, protein, lipid and ash levels in the test ingredients were determined using standard methods AOAC (2000). The samples were homogenized and dried at 105 °C by 24 h prior to the chemical analyses. The level of crude protein was determined using micro-Kjeldahl method by Labconco System (Labconco, Kansas City, MO). The lipid content was analyzed using a micro Foss Soxtec Avanti 2050 Automatic System (Foss Soxtec, Hoganas, Sweden) after extraction with petroleum ether and ash content was determined by calcination of the samples in a muffle furnace at 550 °C (Fisher Scientific International, Inc. Pittsburgh, PA, USA). NFE was determined by the difference between sums of all nutrients.

In vitro degree of hydrolysis (DH)

Digestibility of 13 different protein sources was evaluated by *in vitro* pH-Stat system Tritando Meltrohm 901, where list of ingredients used is summarized in Table 1. *In vitro* hydrolysis assays were performed with crude extracts from stomach (St) or pyloric caeca-intestine (PC-I) only in early and late juvenile stages. To determine protein degree of hydrolysis (DH), every single protein source was incorporated in a concentration of 8 mg ml⁻¹ to be used as substrate solution, according to Saunders *et al.* (1972) and modified by Dimes & Haard (1994). For both juvenile stages, St extracts were adjusted to be added in

substrate solution at 193 U mL⁻¹ and start acid hydrolysis at pH 3.0 in continuous agitations for 15 min (900 s) at 37 °C. Hydrochloric acid (HCl 0.1N) spent to maintain constant pH 3.0, was recorded every 100 seconds. Alkaline hydrolysis degree was performed adding PC-I pool extracts. As previously described, extracts from PC-I were adjusted to be added in the substrate solution at 23 U mL⁻¹ and start alkaline hydrolysis at pH 8.0 in continuous agitation for 45 min (2700 s) at 37 °C. Sodium hydroxide (NaOH 0.1N) spent to maintain constant pH 8.0 was recorded every 250 seconds. All assays were performed by triplicated and procedure was performed for both juvenile stages under same parameters. The DH was calculated using the algorithm according to Adler-Nissen (1986).

During pH-Stat hydrolysis reaction, samples of mixture reactions (40 µl) were collected every 100s for acid hydrolysis reaction and every 250s for alkaline hydrolysis reaction to perform amino acid quantification analysis.

Table 1. Nutrient composition of protein sources used in assays

PROTEIN SOURCE	Abbreviation	%PROTEÍN	%LIPIDS	%ASH	%NFE
Casein ^a	Cas	90	1.2	----	----
Hemoglobin ^b	Hm	90	< 1	----	----
Fish meal ^c	FM	70.7	9.0	12.9	7.41
Tuna by products meal ^d	TM	59	14.9	22.4	3.61
Krill meal ^e	KM	56.7	19.6	9.6	14.1
Squid meal ^e	SM	68.5	2.6	11.6	17.3
Meat porcine meal ^f	MPM	59.7	10.7	12.8	16.8
Meat and bovine meal ^f	MBM	49	13.8	25.1	12.1
Poultry by products meal ^f	PM	61.6	15.3	10.4	12.7
Wheat gluten meal ^g	WGM	81.1	0.73	1.2	16.9
Corn gluten meal ^g	CGM	72.7	3.4	1.4	22.5
Soybean meal ^f	SBM	47.3	0.66	7.0	45.0
Canola meal ^f	CM	42.8	2.1	7.2	47.8
Control diet ^h	D-Control	45.5	10.5	9.9	34.1

^aHammarsten quality Casein, Research Organics # Catalog 1082C, ^bBovine erythrocytes US Biological # Catalog H1850, ^cPremium grade fish meal was obtained from Selecta de Guaymas, S.A. de C.V. Guaymas, Sonora, México, ^dMaz Industrial, S.A de C.V. Mazatlán, Sinaloa, México, ^ePROAQUA, S.A. de C.V. Mazatlán, Sinaloa, México, ^fProteínas marinas y agropecuarias S.A. de C.V., Guadalajara, Jalisco, ^gDroguería Cosmopolita, S.A. de C.V. México, D.F., México, ^hDiet manufactured in CIAD for snapper feeding as a reference diet.

Total amino acid release (TAAR)

Total amino acids (AA) released analysis was performed according to Church *et al.* (1893). An *o*-phthalaldehyde (OPA) solution was prepared with 50 ml of sodium tetraborate 100 mmol l⁻¹, 5 ml of SDS at 20%, 80 mg of OPA diluted in 1ml of methanol and 0.2 ml of β-mercaptoethanol, solution was mixed and brings to 100 ml with distilled water. Briefly, 20 µl of the samples collected in digestion mixture reactions were fixed in 20 µl of 12% TCA and centrifuged at 14000 rpm during 15 min. Supernatant samples of 10 µl were added to 1 ml of OPA solution and absorbances were read at 340 nm. TAAR was calculated using standard curve made with decrees L-leucine concentrations.

Zymogram analyses

Electrophoresis techniques were performed in Mini PROTEAN 3 Cell (Bio- Rad) with four plates vertical gels of 8x10x0.075 cm with 10 sample capacity per plate. For the analysis of acid proteases from stomach, electrophoresis was run under non-denaturing native conditions (Native-PAGE) composed by continuous acrylamide gel (10 %) in buffer Tris (25 mmol l⁻¹) and glycine (192 mmol l⁻¹, pH 8.3, 80 volts) according to Davis (1964). Plate was composed by stacking gel with 4% poly-acrylamide (PAA) and resolving gel with 10% PAA. Electrophoresis was run under denaturalizing conditions (SDS-PAGE), with SDS 0.1 % in buffer Tris (25 mmol l⁻¹) and glycine (192 mmol l⁻¹, pH 8.3, 100 volts), according to Laemmli (1970) and adapted by García-Carreño *et al.* (1993).

After Native-PAGE electrophoresis, the gels were treated to reveal proteases isoforms according to the procedure of Díaz-López *et al.* (1998). The gel was submerged for 90 min at 25 °C in solution containing 0.25% hemoglobin (0.1 mol l⁻¹ Glycine-HCl buffer, pH 2.0). The gels were fixed in trichloroacetic acid (12%) solution by 15 minutes. After alkaline SDS-PAGE electrophoresis, the gels were washed and directly incubated for 30 min at 5 °C in 0.5% casein solution (Tris-HCl 0.1 mol l⁻¹ buffer, pH 9). The gels were then incubated for 90 min in the same solution at 37 °C. Finally, the gels were washed and fixed as previously described. For acid and alkaline gels, after areas of enzyme activity had been developed, the gels were stained according to Weber and Osborn (1969), using 0.1% Coomassie brilliant blue R-250 solution. Electrophoretic techniques were complemented

with the use of specific inhibitors previous described. Molecular weight marker was applied to each SDS-PAGE. Molecular weight (MW) of each band in the SDS-zymograms (alkaline protease) was calculated by a linearly adjusted model between the Rf and the decimal logarithm of MW protein markers.

Statistical analysis

For comparison, the percent inhibition and percent of relative activity in enzyme characterization and pH-Stat degree of hydrolysis was arcsin ($x^{1/2}$) transformed. The data for each parameter were tested for normality and homoscedasticity. One- or two-way ANOVA analyses were run when required. When differences were found, Tukey's HSD test was used ($P \leq 0.05$). Total amino acids released (mg L-Leucine equivalent) was plotted describing relationship between cumulative amino acid release and time of digestion for different meals with linear adjustment ($y = a + bx$). Differences among rate of digestion (slopes) between protein sources in specific hydrolysis phase and juvenile stage were assessed with ANCOVA ($P \leq 0.05$) (Zar 1984). All of the statistical analyses were performed using Statistica 7.0 Software for Windows (StatSoft, USA).

Results

Enzyme activity assays

The acid and alkaline proteases activities of different digestive tract sections in three juvenile stages are presented in Table 2.

Table 2. Protease activity in the stomach (S), pyloric caeca (PC), proximal (PI), middle (MI) and distal intestine (DI) in three juvenile stages of spotted rose snapper *Lutjanus guttatus*.

Stage	Specific Activity (U mg protein ⁻¹) of crude extract				
	ST	PC	PI	MI	DI
EJ	1754.4±307.8 ^c	17.4±5.9 ^b	15.0±1.1 ^c	15.6±2.9 ^b	15.8±3.2 ^c
MJ	3864.2±796.0 ^b	22.2±3.8 ^b	20.0±2.4 ^b	27.5±5.0 ^a	23.0±3.8 ^b
LJ	6210.1±657.6 ^a	32.3±4.2 ^a	28.2±3.0 ^a	29.1±6.4 ^a	34.0±6.2 ^a

The stomach acid proteolytic activity showed significantly higher specific activities ($P \leq 0.001$) value with increasing life stage. No significant differences in specific activity of alkaline proteases were observed between pyloric caeca and intestine sections for all juvenile stages ($P \leq 0.001$). Meanwhile, significantly higher specific activities in the LJ stage ($P \leq 0.001$) were found between stages when individual sections were compared. The trypsin-like specific activity showed a significantly higher ($P \leq 0.001$) value in the EJ stage than MJ and LJ stages (Table 3).

Table 3. Trypsin-like activity in the pyloric caeca in three juvenile stages of spotted rose snapper *Lutjanus guttatus*. Different superscript within rows indicate significant differences ($P < 0.05$).

Specific Activity (U mg protein ⁻¹)		
EJ	MJ	LJ
82.50±2.24 ^a	23.18±2.47 ^b	22.77±9.66 ^b

Temperature and pH effect on acid and alkaline protease activity

The three juvenile stages presented optimum temperature of acid proteases at 45°C (Fig. 1A) ($P \leq 0.001$). The optimum temperature of total alkaline proteases was 55°C for EJ, 50°C for MJ and LJ (Fig. 1B) ($P \leq 0.001$).

The optimum activity of acid proteases was measured at pH 3 for EJ and LJ and at pH 2 for MJ, with 80 to 90% of remnant activity at pH 2 and 3, respectively (Fig. 1C) ($P \leq 0.001$). Alkaline protease activity showed high relative activity (%) over a wide pH range (5-10) and an optimum at pH 9.0 in the three juvenile stages (Fig. 1D) ($P \leq 0.001$). Differences were found in relative activity percent at pH 5 between LJ (80%) and EJ, MJ (50%) ($P \leq 0.001$).

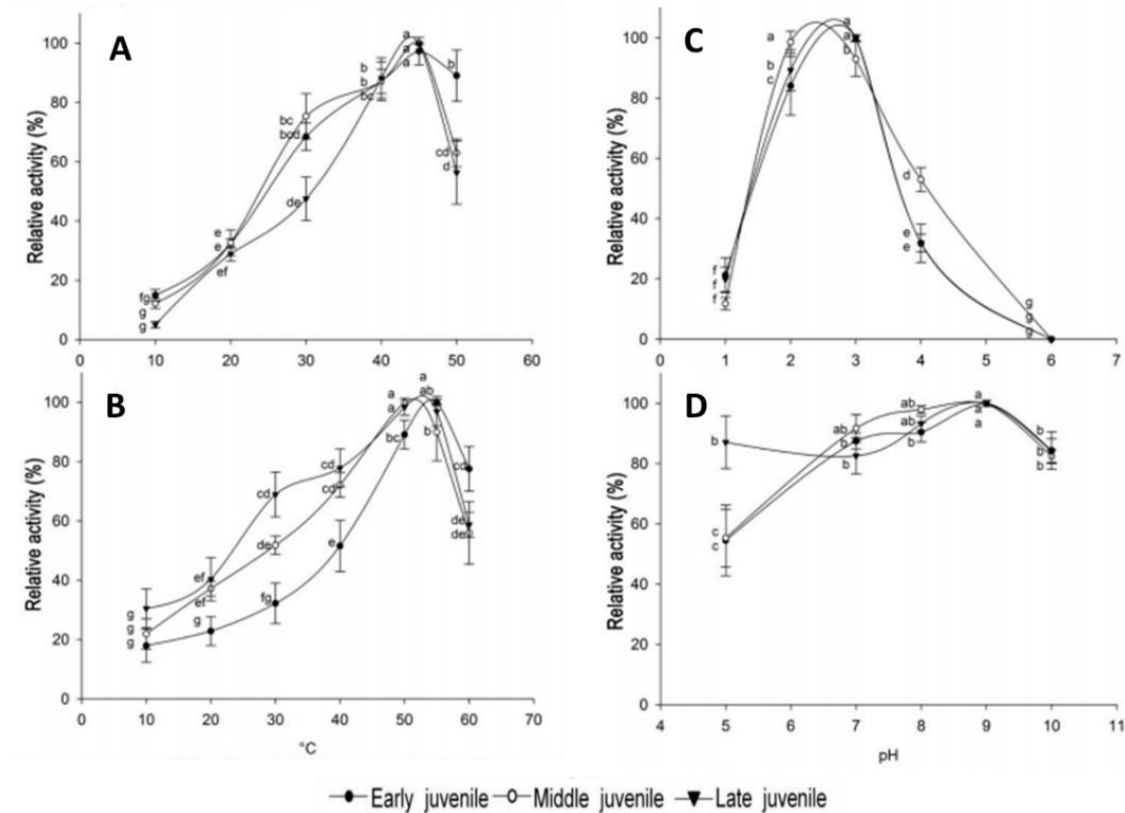


Figure 1. Temperature effects (°C) on the relative activity of acid (a) and alkaline proteases (b) and pH effects on the relative activity of acid (c) and alkaline proteases (d) in three juvenile stages of *Lutjanus guttatus*.

Temperature and pH effect on trypsin activity

The optimum temperature of trypsin was 50 °C for MJ and LJ, while EJ presented an optimum at 60 °C. Differences were found in relative activity (%) between almost all temperatures tested ($P \leq 0.001$) (Fig. 2B). Trypsin activity showed optimum activity at pH 9 for all juvenile stages (Fig. 2B).

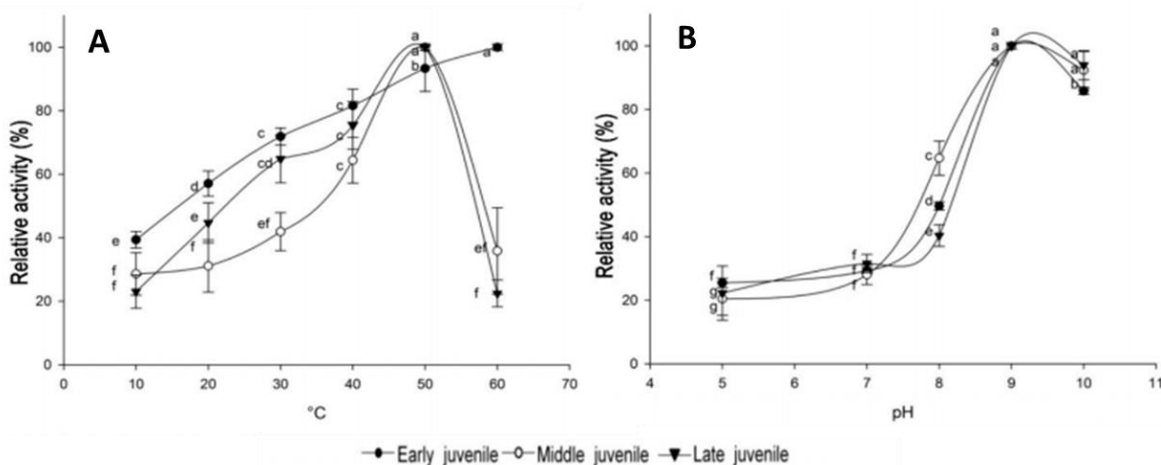


Figure 2. Temperature and pH effects on the relative trypsin-like activities in three juvenile stages of *Lutjanus guttatus*.

Specific inhibitors effects

Pepstatin A inhibited the total activities in stomach extracts in all juvenile stages. The percent of alkaline protease inhibition are summarized in Table 4. In general, the inhibited percent of activity in total alkaline proteases was significantly higher ($P \leq 0.001$) in EJ using TLCK, PMSF, SBTI, Phen and Ovo compared to MJ and LJ, while no significant differences were found between inhibition percent with TPCK ($P = 0.2402$).

Table 4. The percent of activity inhibition in pyloric caeca after incubation with enzyme specific inhibitors in three juvenile stages of spotted rose snapper *Lutjanus guttatus*.

Inhibitor type	Percentage of activity inhibition					
	TPCK	TLCK	PMFS	SBTI	Phen	Ovo
EJ	11.7±4.8 ^a	14.2±1.3 ^a	15.7±2.5 ^a	54.9±6.6 ^a	32.7±2.0 ^a	18.5±1.2 ^a
MJ	9.9±2.6 ^a	6.1±0.6 ^b	13.6±0.6 ^a	25.8±5.4 ^b	28.8±1.3 ^b	7.3±0.5 ^b
LJ	6.6±2.1 ^a	7.9±1.3 ^b	5.4±1.9 ^b	16.1±3.9 ^c	23.3±1.1 ^c	6.3±1.0 ^b

Different superscript within columns indicate significant differences ($P < 0.05$).

In vitro degree of hydrolysis (DH)

Hemoglobin presented the highest DH among all ingredients in acid digestion for both juvenile stages ($P \leq 0.05$). Higher DH values differ in protein source between juvenile stages, where SBM (soybean meal), CM (canola meal) and D-control (control diet) showed

the higher DH values in LJ acid digestion, while TM (tuna by products meal), SBM and D-control showed the highest DH values in EJ acid digestion. By the other side, DH values of TM, SM (squid meal) and CM showed differences between EJ and LJ stages in acid hydrolysis ($P \leq 0.05$) (Fig. 3A).

Alkaline hydrolysis showed that FM (fishmeal) presented the higher degree of hydrolysis among all ingredients in LJ stage, while MBM (meat and bovine meal), WGM (wheat gluten meal), CGM (corn gluten meal) and D-control presented the highest DH among all ingredients in EJ stage ($P \leq 0.05$). Eight of the ingredients tested, showed differences in DH between EJ and LJ stages in alkaline hydrolysis, including animal protein sources (FM, MPM (meat porcine meal), MBM, PM (poultry by products meal)) and vegetable protein sources (WGM, CGM, CM) and D-control, as protein mix from balance diet ($P \leq 0.05$) (Fig. 3B).

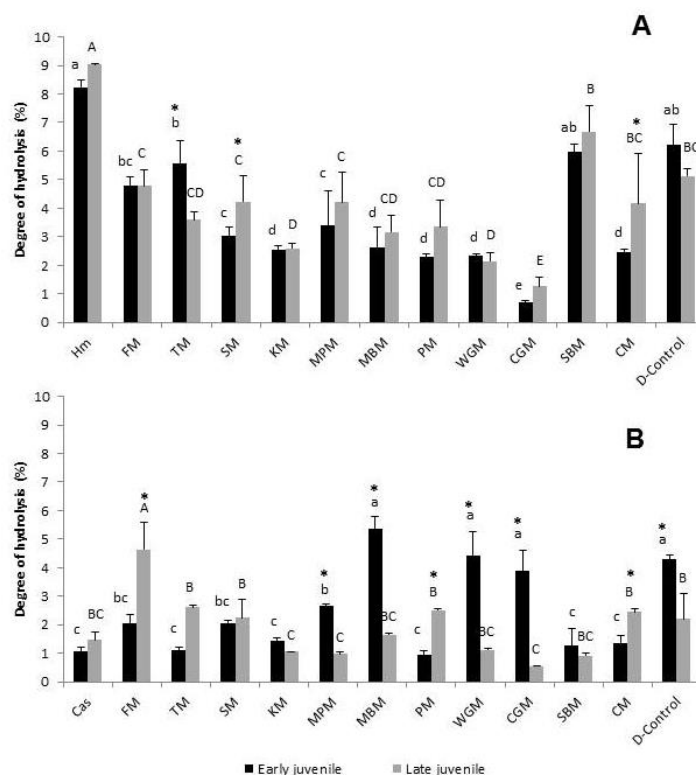


Figure 3. *In vitro* pH-stat degree of protein hydrolysis (DH) of feed ingredients using digestive enzyme extracts from *L. guttatus* early (20 g) and late juveniles (400g) from A) stomach and B) pyloric caeca. Lower-case show differences in EJ stage, upper-case show differences in LJ stage and asterisk show differences between juvenile stages ($P < 0.05$).

Values shown are means ($n=3$) \pm standard deviation (error bars).

Total amino acid release (TAAR)

The kinetics of TAAR was assessed by analyzing the cumulative production of amino acid through time of digestion. These relationships were best described by linear regressions, all of them with high determination coefficients ($R^2=0.90$ to 0.98). The rate of amino acid liberation were compared by ANCOVA and showed significant differences between ingredients in acid and alkaline hydrolysis in both juvenile stages ($P \leq 0.05$).

For both juvenile stages, hemoglobin presented the highest TAAR. Nevertheless, higher TAAR with stomach extracts in EJ stage was obtained by SM, followed by CM, SBM and KM (krill meal), while TM showed the lowest TAAR ($P \leq 0.05$) (Fig. 4A).

Peña, E. et al., 2017. Change in protein digestion capacity during juvenile fish ontogeny: Approach on spotted rose snapper (*Lutjanus guttatus*). En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. (Eds), Investigación y Desarrollo en Nutrición Acuicola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 408-431. ISBN 978-607-27-0822-8.

Results in TAAR by LJ stomach extract show higher values for SBM and CM, followed by MBM and WGM, while CGM showed the lowest TAAR ($P \leq 0.05$) (Fig. 4B).

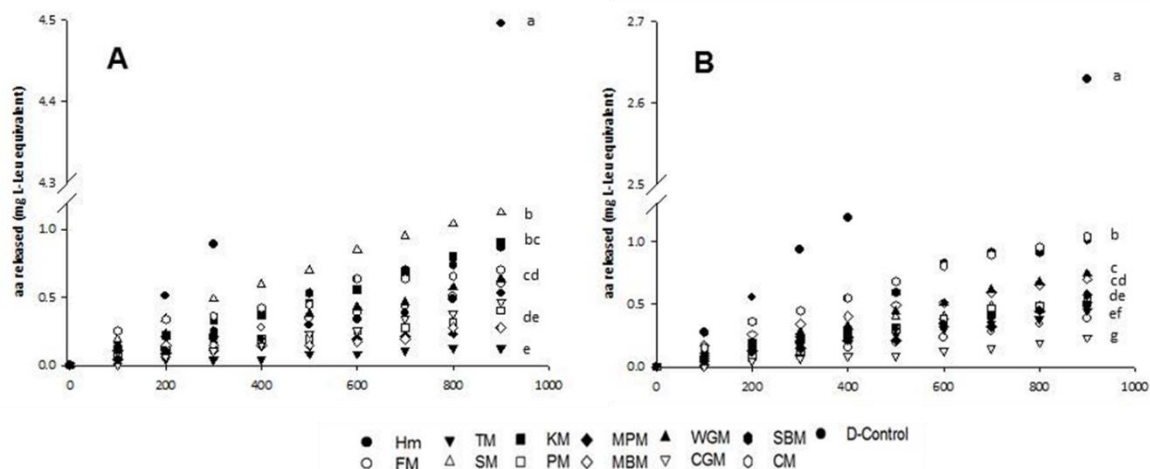


Figure 4. Kinetic of free amino acid released from ingredients using stomach enzyme extracts from *L. guttatus* a) early (20 g) and b) late juveniles (400g). Data points and regression lines of cumulative values against time for each meal are represented with the same symbol. Letters to the right of regression lines indicate differences ($P \leq 0.05$) among slopes.

Alkaline hydrolysis in EJ stage showed higher TAAR by PM, followed by Cas (casein), MPM and TM, while SBM and CGM showed the lowest TAAR ($P \leq 0.05$) (Fig. 5A). Alkaline hydrolysis in LJ stage showed higher TAAR in MPM, followed by FM, while Cas and SBM showed the lowest TAAR values ($P \leq 0.05$) (Fig. 5B).

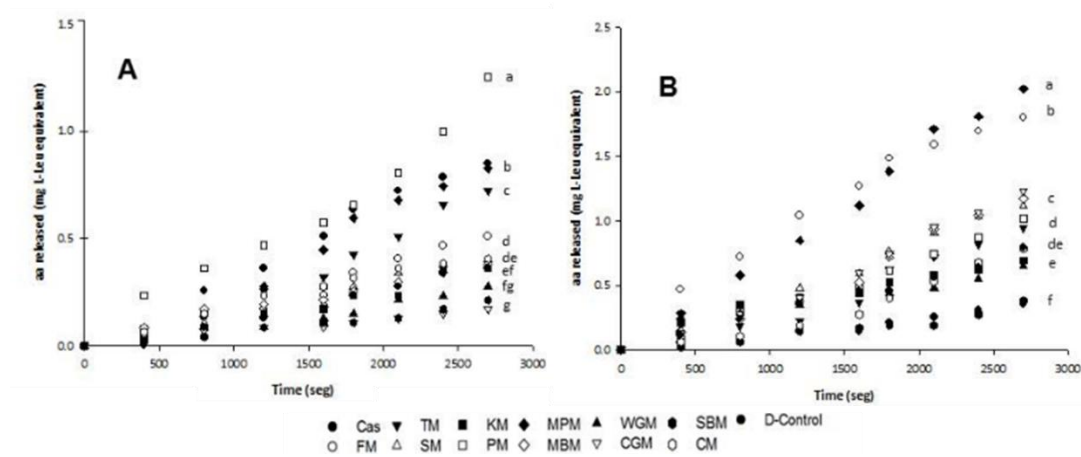


Figure 5. Kinetic of free amino acid released from ingredients using pyloric caeca-intestine enzyme extracts from *L. guttatus* a) early (20 g) and b) late juveniles (400g). Data points and regression lines of cumulative values against time for each meal are represented with the same symbol. Letters to the right of regression lines indicate differences ($P \leq 0.05$) among slopes.

Zymogram analyses

Electrophoresis under Native-PAGE conditions, reveal two bands with acid protease activity in both juvenile stages of SRS: one with an R_f of 0.72 and the other with an R_f of 0.77, where both bands were completely inhibited by pepstatin A (Fig. 6).

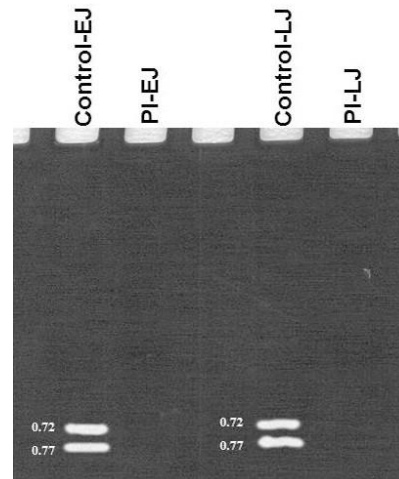


Figure 6. Zymogram of acid proteases from the multienzymatic stomach extracts of early juvenile (EJ; 20g) and late juvenile (LJ; 400g) stages of *L. guttatus*, with the action of pepstatin A inhibitor (PI) on the isoforms.

Electrophoresis under SDS-PAGE conditions showed same band pattern in pyloric caeca and intestine sections, therefore results corresponds to all alkaline phase digestive tract in *L. guttatus* in a given juvenile stage. Total of nine bands in PC-I extracts were observed between EJ and LJ stages bands (Fig. 7A and Fig. 7B, respectively; 98.1, 90.2, 87.3, 71.4, 53.1, 40.6, 26.1, 19.8 and 16.7 kDa), referenced as first to ninth bands.

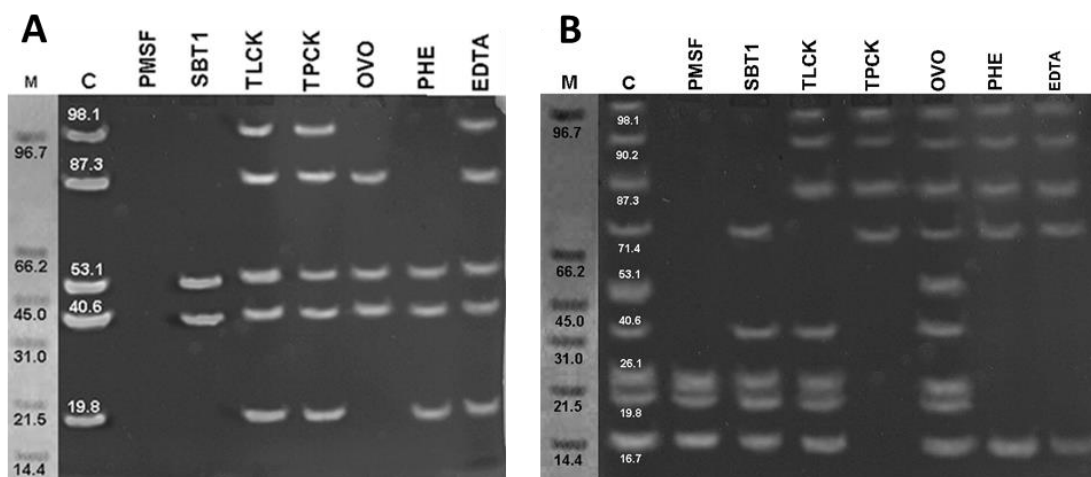


Figure 7. Zymograms of alkaline proteases from the multienzymatic pyloric caeca and intestine extracts of A) Early juvenile of *L. guttatus* (20 g) and B) Late juvenile of *L.*

guttatus (400 g), with the action of the respective inhibitors on the isoforms. M: molecular weight marker (kDa).

In the case of alkaline enzyme pattern in EJ stage, five bands were observed in the control (98.1, 87.3, 53.1, 40.6 and 19.8 kDa, representing, first, third, fifth, sixth and eight bands) (Fig. 7A). Four additional bands were observed in LJ stage PC-I extracts, with MW of 90.2, 71.4, 26.1 and 16.7 (representing the second, fourth, seventh and ninth bands) with a total of nine bands (Fig. 7B).

Discussion

Although there are many investigations in characterization of digestive enzymes in different fish species, much of this research has focused on early ontogeny and / or characterization in a juvenile size of this species. During the early ontogeny of many fish species, there are successive changes in the activity and / or expression of different enzymes together with a rapid development of the digestive system and auxiliary organs (Zambonino-Infante & Cahu, 2001), that is helpful to close cultivation cycles of species with aquaculture potential.

On the other hand, the characterization of digestive enzymes that have been carried out during juvenile stages, take for granted that the number, type and / or activities of digestive enzymes do not change throughout the juvenile stage, where some studies have approached this subject in some species (Unajak *et al.* 2012, Yasumaru & Lemos, 2014). The presence of changes in digestive capacity during juvenile stages of different species is of great importance, since these stages of life correspond in the majority of the fish aquaculture species to the phase of culture before harvest. Therefore, understanding of digestive changes and / or adaptations will serve as a basis for the formulation of specific diets for grow-out juvenile stages, in order to increase the productive yield.

Thus, the present investigation serves as a basis for the development of research in different species with culture potential, where it is clear the existence of modifications in the digestive capacity of proteins in a same species during juvenile development.

Conclusion

In conclusion, the digestive system of spotted rose snapper is highly efficient in the breakdown of protein. The high pepsin activities and the presence of two pepsin isoforms suggest the potential for hydrolysis of a wide range of protein sources joined to final alkaline digestion. This potential increases with fish growth through juvenile stages in which a diversification in the type of alkaline enzymes exists, affecting the degree of hydrolysis of different protein sources and the rate and degree of absorption of total free amino acids. Higher DH and TAAR values were documented in constituents such as fish and squid meal, animal porcine meal and poultry meal produced from recycled by-products and soybean meal and canola meal as vegetable products that provide better protein sources for use in the development of practical diets.

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Synthetic Growth Hormone Secretagogue GHRP-6 Exhibits Enhanced Growth Activity and Immune System Stimulation in Teleost Fish and Shrimp

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Abstract

Aquaculture is part of the solution for meeting the growing world demand for food, mainly as an animal protein source. However, current yields are insufficient for aquaculture to play this crucial role. Growth-rate enhancement is one of the approaches that have been exploited in this regard. Also, losses caused by bacteria, viruses and parasites remain a significant problem. It has been demonstrated that growth stimulants contribute not only to growth enhancement but also to fish health improvement. Growth Hormone Releasing Peptide-6 (GHRP-6) is one of the earliest-developed, synthetic, peptidyl growth hormone secretagogue receptor agonists. These compounds mimic the effect of the endogenous ligand, ghrelin. This peptide has shown its benefits for both growth enhancement and immune system stimulation in fish and crustaceans. In the present study, we demonstrate that intraperitoneal administration of GHRP-6 induces liver insulin growth factor-I messenger RNA, and increases growth hormone levels in juvenile tilapia (*Oreochromis* sp.), in a time-course experiment. In addition, administration of GHRP-6 in formulated feed to tilapia larvae was assessed. Growth and immune parameters such as lectin titers and intestinal intraepithelial lymphocyte numbers were increased in treated larvae. We also evaluated the effect of GHRP-6 injection over feed intake in shrimp and its effects on shrimp growth when the peptide was administrated by successive immersion baths. GHRP-6 increased feed intake, body weight and size, the number of rostral spines and gill branches, protein concentration and haemocyte number in treated shrimps.

Keywords: Growth Hormone, Secretagogues, Peptides, Larvae, growth, Innate Immune state

Introduction

Aquaculture is part of the solution for meeting the growing world demand for food, mainly as an animal protein source. However, current yields are insufficient for aquaculture to play this crucial role. Growth-rate enhancement is one of the approaches that have been exploited in this regard. Also, losses caused by bacteria, viruses and parasites remain a significant problem. It has been demonstrated that growth stimulants contribute not only to growth enhancement but also to fish health improvement.

In teleosts fish, secretion of GH is regulated by several hypothalamic factors that are influenced by the physiological state of the animal. There is an interaction between immune and endocrine systems through hormones and cytokines. GH in fish is involved in many physiological processes that are not overtly growth related, such as saltwater osmoregulation, antifreeze synthesis, and the regulation of sexual maturation and immune functions. (Devlin *et al.* 2009)

The synthetic growth hormone (GH) secretagogues (GHSs) consist of a family of ligands, first described by Momany *et al.*, (1981). They were initially termed GH-releasing peptides (GHRPs). These synthetic compounds were developed to release GH *in vitro*. They were predicted to mimic the effect of an endogenous factor that would activate a specific receptor in the pituitary and the hypothalamus. The cloning of the receptor for these non-classical GH-releasing compounds, together with the more recent characterization of the endogenous ligands, ghrelin and des-Gln14-ghrelin, has unambiguously demonstrated the existence of a physiological system that regulates GH secretion along with GHRH and somatostatin.

Methodology

In this work we characterized for first time the GHRP-6 action over fish and crustacean growth and immunity. GHRP-6 is one of the earliest-developed, synthetic, peptidyl growth hormone secretagogue receptor agonists. It was described by Bowers *et al.* 1984.

Our research group demonstrated that GHRP-6 increased pituitary GH secretion *in vitro* (Lugo *et al.* 2008; Martinez *et al.* 2012). In addition, food intake increased when GHRP-6 was administered by intraperitoneal injection to juvenile tilapia (Lugo *et al.* 2010), and body weight gain when given by immersion baths to tilapia larvae (Martinez *et al.* 2012). We also demonstrated that intraperitoneal administration of GHRP-6 induces liver insulin growth factor-I messenger RNA, and increases growth hormone levels in juvenile tilapia (*Oreochromis sp.*), in a time-course experiment. GHRP-6 also stimulates growth using non-encapsulated and encapsulated peptide administered by a plastic tube to the pharyngeal cavity to juveniles. In addition, administration of GHRP-6 in formulated feed to tilapia larvae was assessed and growth stimulation by this administration route demonstrated.

Results and Discussions

Growth and immune parameters such as lectin titers and intestinal intraepithelial lymphocyte numbers were increased in treated larvae.

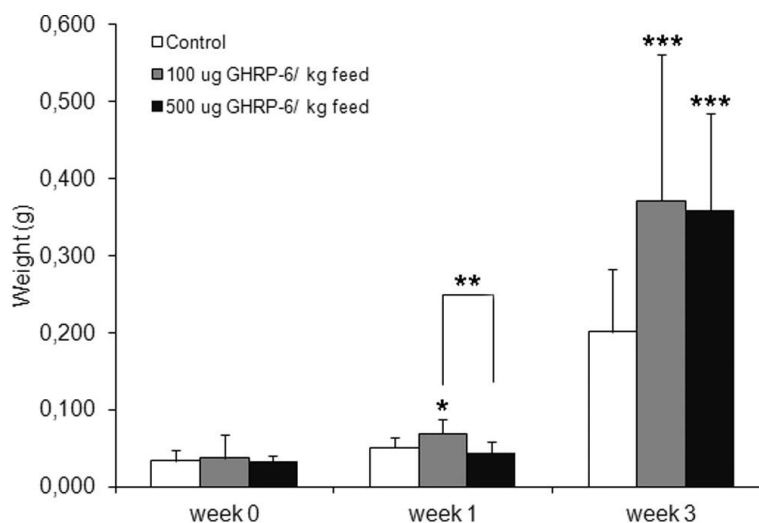


Figure 1. Growth experiment in tilapia larvae fed with GHRP-6. Two different doses were assayed: 100 µg of GHRP-6/kg feed and 500 µg of GHRP-6/kg feed. A control group was fed with the same feed but without the peptide. GHRP-6 growth-promoting effects were evaluated by measuring the body weight increase at week 1 and 3. Data are represented as mean + standard deviation. Data were analyzed by Kruskal–Wallis test followed by Dunn's Multiple Comparison Test. Asterisks represent statistically significant differences: (*) p b 0.05; (**) p b 0.01; (***) p b 0.001.

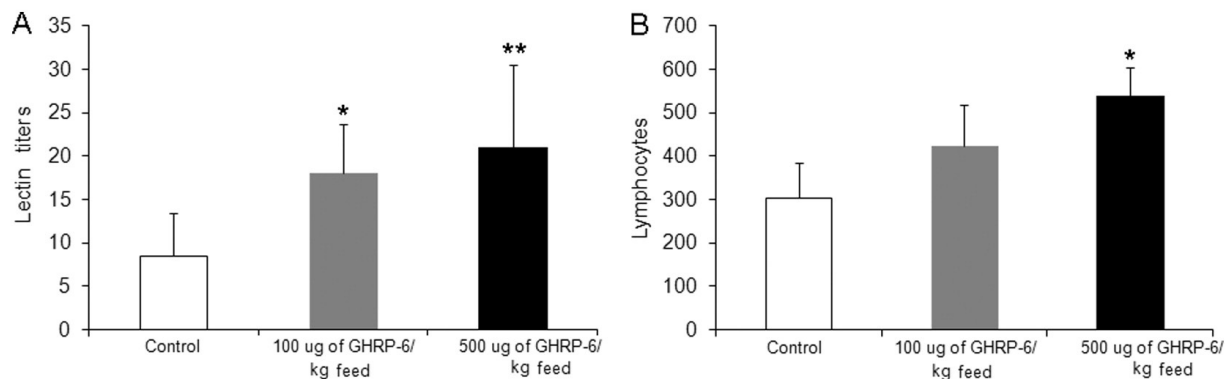


Figure 2: Immune parameters in larval homogenates at 21 days from the beginning of the experiment: Hemagglutination titers for lectins (A) and intraepithelial lymphocytes (B). Two different doses were assayed: 100 μg of GHRP-6/kg feed and 500 μg of GHRP-6/kg feed. A control group was fed with the same feed but without the peptide and treated under similar conditions. Data are represented as mean + standard deviation.

Data were analyzed by Kruskal–Wallis test followed by Dunn's Multiple Comparison Test. Asterisks represent statistical significant differences: (*) $p < 0.05$; (**) $p < 0.01$

GHRP-6 has also a capacity of to boost the immune response to co-injected antigens in tilapia and catfish. Modern subunit vaccines have excellent safety profiles and improved tolerability, but do not elicit strong immune responses without the addition of adjuvants. Adjuvants are substances added to vaccine formulation that are intended to enhance the humoral and/or cell-mediated immune response to the antigen. They can be used to enhance the magnitude and the type of the antigen –specific immune response As far as we know our results demonstrate for first time the adjuvant effect caused by GHRP-6 to enhance the immune response in fish .

Ghrelin has been studied mainly in vertebrates; thus, little is known about its role in invertebrates, including crustaceans. We first evaluated the effect of GHRP-6 injection over feed intake in shrimp and its effects on shrimp growth when the peptide was administrated by successive immersion baths. GHRP-6 increased feed intake, body weight and size, the number of rostral spines and gill branches, protein concentration and haemocyte number in treated shrimps.

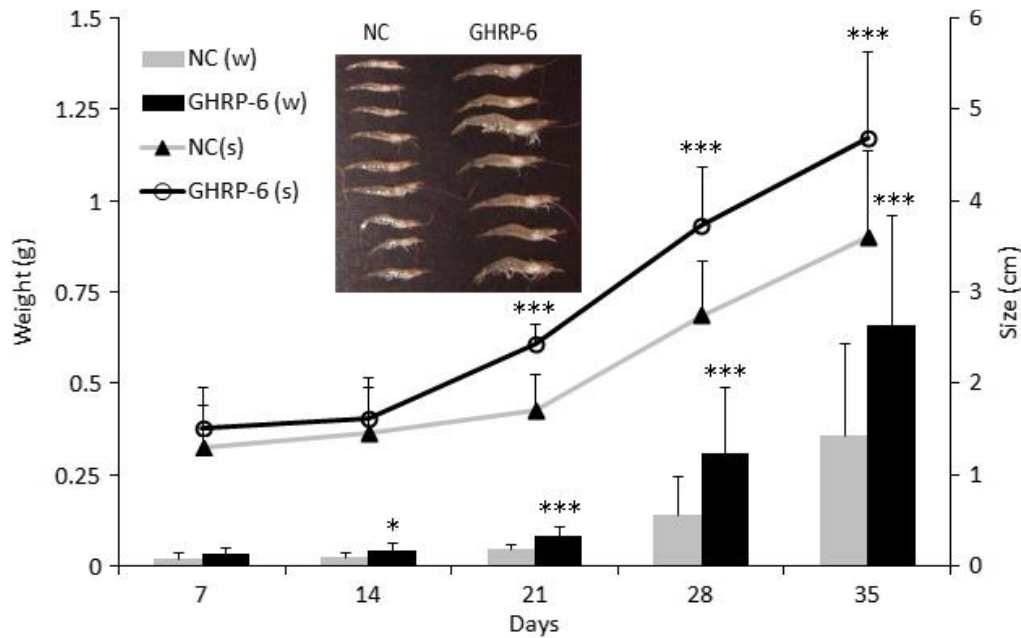


Figure 3. Growth experiment in stage 15 *Litopenaeus vannamei* postlarvae immersed in the ghrelin analogue GHRP-6 over a 35-day period. Left axis: body weight (w in the legend) in grams (g). Right axis: size (s in the legend) in centimetres (cm). The picture on top corresponds to samples at 35 days of treatment. Samples of 50 animals were weighed and sized at 0, 7, 14, 21, 28 and 35 days. Data are represented as mean + standard deviation. GHRP-6-treated group received 200 μ g of GHRP-6 per litre of water (200 μ g L⁻¹), and non-treated group received only the reduction in water volume similar to the treated group. Asterisks represent statistical significant differences: *(P < 0.05); ***(P < 0.001). A Mann–Whitney test was performed.

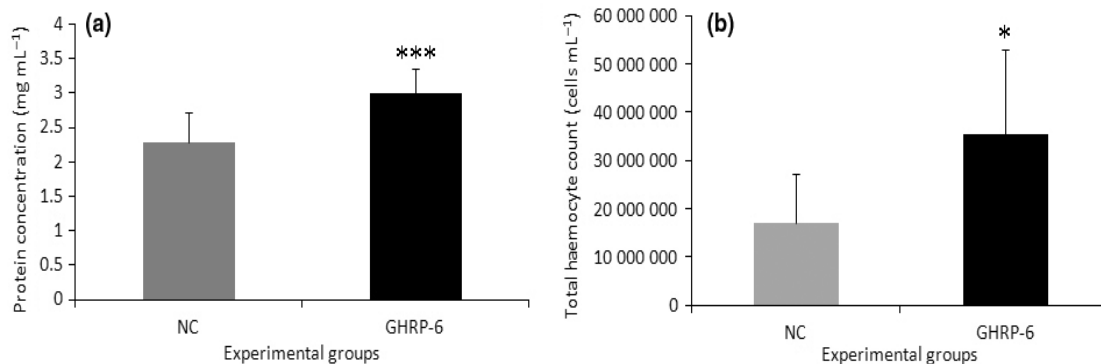


Figure 4. (a) Total protein concentration in shrimp homogenates. (b) Total haemocyte number in the shrimp haemolymph at 35 days of the *Litopenaeus vannamei* growth experiment. GHRP-6-treated group received 200 μ g of GHRP-6 per litre of water (200 μ g L⁻¹), and non-treated group received only the reduction in water volume similar to the treated group. Data are represented as mean + standard deviation, N = 10. Asterisks represent statistical significant differences: *(P < 0.05), ***(P < 0.001). An unpaired t-test was performed.

We also evaluated the peptide uptake and clearance in a pharmacokinetics, using [H3] GHRP-6 administered to postlarvae. Given a limited exposure and efficient clearance of the peptide-associated radioactivity from larvae, our findings suggested that GHRP-6-treated *Litopenaeus vannamei* can be consumed safely by humans after aquaculture applications. These results propose that GHRP-6 could be an additional tool to study growth physiology in crustaceans and also a promising candidate for development into a new biotechnology product for improving shrimp and fish growth and quality.

Scientific Support:

Article 1

Oral administration of the growth hormone secretagogue-6 (GHRP-6) enhances growth and non-specific immune responses in tilapia (*Oreochromis* sp.). Rebeca Martínez, Yamila Carpio, Antonio Morales, Juana María Lugo, Fidel Herrera, Claudina Zaldívar, Olimpia Carrillo, Amílcar Arenal, Eulogio Pimentel, Mario Pablo Estrada. **Aquaculture** 452 (2016) 304–310.

Article 2

Significant improvement of shrimp growth performance by growth hormone-releasing peptide-6 immersion treatments. Rebeca Martínez*, Yamila Carpio*, Amílcar Arenal, Juana María Lugo, Reynold Morales, Leonardo Martín, Ramón Franco Rodríguez, Jannel Acosta, Antonio Morales, Jorge Duconge, Mario Pablo Estrada. (*) Both authors contribute equally to this work. **Aquaculture Research**. (2017)

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Protein and Amino Acid Nutrition of Marine Fish Species

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Abstract

Intensive aquaculture of marine fish species has highly expanded during the last decades and has potential for further increase. An increased and consistent supply of high quality aquafeed will be required to sustain this production and to guaranty the production of protein of high biological value for humans. Protein is the most abundant and expensive dietary nutrient in aquafeeds and fish meal is still the major dietary protein source in marine aquafeeds. Given the zootechnical, environmental and economical importance of a well-balanced diet, fine-tuning of dietary protein and amino acid composition to closely meet fish requirements are therefore of utmost importance. This is of particular importance when it is considered the dietary replacement of fish meal by more sustainable and renewable protein sources, such as plant feedstuffs, which have amino acids unbalances, antinutritional factors, lower protein content and digestibility, and devoid or containing very low concentrations of some particular micronutrients, as taurine. Concomitantly, in fish the utilization of amino acids for energy purpose is considered high, and so optimization of amino acids/protein accretion has great importance and practical implications. Besides the nutritional proprieties of amino acids, emerging evidence shows that some of them have functional properties, regulating key metabolic pathways crucial to maintenance, growth, and immune responses, being of strong interest in fish production. The importance of gaining further knowledge regarding amino acid requirements, metabolism and utilization as functional ingredients in aquaculture fish species will be stressed.

Keywords: Amino acids; Aquaculture; Marine fish; Nutrition; Protein; Requirement

Introduction

Intensive aquaculture is a feed-based production system and, therefore, improved diet specification is needed to reduce cost and increase productivity while guarantying the production of high biological value protein for humans. To address this, critical information is required on protein requirement of the target species at different phases of life cycle. Indeed, protein is one of the most expensive dietary component and a major factor influencing fish growth performance, feed cost and nitrogen pollution, and thus of the productivity and environmental sustainability of farmed fish. A deficient supply of dietary protein leads to decreased growth and therefore economic production loss, but provided in excess of requirements, protein is catabolized and used for energy purposes, increasing the nitrogen load to the environment. The knowledge on species protein and essential amino acid (EAA) requirements and optimum dietary EAA profile is therefore of particular importance for the formulation of cost-effective diets aiming to maximize growth and protein retention while minimizing nitrogen excretion. This data is also a pre-requisite for the correct evaluation of alternative protein sources to fishmeal in order to reduce the actual dependency of aquafeed of this commodity. Apart from low digestible protein content of most plant feedstuffs, plant protein feedstuffs also have deficiencies in certain essential amino acids such as lysine, methionine and arginine and functional amino acids as taurine and hydroxyproline (Aksnes *et al.*, 2008; Zhang *et al.* 2013; Salze & Davis, 2015). Thus, dietary AA deficiencies/unbalance may occur when plant feedstuffs are used as the main dietary ingredients, thus reducing growth and feed utilization (Gatlin *et al.* 2007). It is likely that production of non-fishmeal diets, capable of supporting growth rates necessary for the economic production of farmed fish, will require the adoption of more modern formulation approaches, taking into account nutrient availability, especially in regard to EAA, and combining different plant feedstuffs and EAA supplementation to meet the required EAA for the species (Nunes *et al.* 2014).

Protein requirements

Fish species requires high dietary protein levels, 2-4 times higher than those of farm animals, although in absolute terms (protein per unit of weight gain) requirements are not higher than those of farm animals (Table 1; Bowen, 1987). The higher protein content of fish diets might be incorrectly attributed to the carnivorous feeding habits of fish; however, it is mostly due to the differences in energy requirements between fish and farm animals. Fish are heterothermic animals, and therefore do not spend much energy for maintenance. Energy requirements for maintenance is circa 10-20 times lower in fish than in farm animals, which homoeothermic animals, and therefore spend large amounts of energy to maintain body temperature (Cho and Kaushik, 1990). Moreover, fish also have low maintenance protein requirements, representing only 11 to 35% of the total protein needs (Cho and Kaushik, 1990; McGoogan and Gatlin, 1998; Lupatsch *et al.* 2001; 2003; Dias *et al.* 2003; Peres and Oliva-Teles, 2005; Ozório *et al.* 2009). Thus, dietary protein content in relation to the dietary energy density is higher in fish than in farm animals, this explaining their apparently high dietary protein requirements.

Table 1. Growth performance, diet and protein utilization efficiency of fish and terrestrial animals¹.

	Fish	Terrestrial animals
Specific growth rate	2.77	2.45
Dietary protein content (%)	40.3	20.0
Daily protein intake at maximum growth (mg Prot/g BW/day)	16.5	12.0
Protein retention (% protein intake)	31.0	29.0
Protein efficiency ratio	1.95	1.97
Feed conversion efficiency	0.78	0.26

¹Adapted from Bowen (1987); BW, body weight.

Dietary protein requirements of marine fish species have been investigated mostly based on conventional dose-response studies, yielding minimum protein required to attain maximum growth. Studies indicate that dietary protein requirement for maximum growth of marine fish range between 27% and 55% (Table 2). Lower dietary protein requirement is

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usually recorded for omnivorous species, such as white seabream and two-banded seabream, than for carnivorous species. These differences in dietary protein requirements may be related either to differences between species in feeding habits or in growth potential (Oliva-Teles *et al.* 2011). Comparatively to salmonids, most carnivorous marine fish species apparently have higher protein requirements and use dietary protein less efficiently. Indeed, protein retention as percentage of intake ranges between 30-40% for salmonids and between 25-35% in marine carnivorous fish (Tibaldi & Kaushik, 2005; Lall and Anderson, 2005). This difference in protein efficiency may have a nutritional basis or may be due to inadequate diet formulation. This being the case, it is expectable that better knowledge of nutritional requirements of marine fish may improve protein utilization efficiency and consequently allow a reevaluation of dietary protein requirements (Tibaldi & Kaushik, 2005).

Dietary protein may be used for plastic or for energy purposes, depending on the overall dietary protein to energy ratio. Moreover, fish are well adapted to metabolize protein and they use it preferentially to lipids or carbohydrates as an energy source (Walton and Cowey, 1982). Thus, optimization of dietary digestible protein to digestible energy (DP:DE) ratio is of crucial importance in improving protein utilization as it will allow sparing protein from energy purposes and use it preferentially for protein synthesis (Cho and Kaushik, 1990). Indeed, DP:DE ratio is a more rational way of expressing protein requirements than dietary crude protein alone (NRC, 2011). For marine fish species, recommendation of optimum dietary DP:DE ratio range from 20 to 32 g DP/MJ DE, depending on species (NRC, 2011). On the other hand, for salmonids optimum dietary DP:DE ratio is 17-18 g DP/MJ DE (Hillestad and Johnsen, 1994; Hillestad *et al.* 1998). This huge difference between groups of fish may be related to the capacity of salmonids of using higher dietary lipid than marine fish (Peres & Oliva-Teles, 1999a; NRC, 2011).

An optimization of the DP:DE ratio, through the reduction of dietary protein level with or without the concomitant increase of the energy level, reduce the utilization of protein to meet energy requirements, reducing ammonia excretion and environmental impact of aquaculture (Cho, 2008; NRC, 2011). In seabass, for example, reduction of dietary protein level from 56 to 48% led to a significant reduction (about 26%) of the relative contribution of protein catabolism to energy expenditure and a reduction (about

28%) of ammonia excretion (Peres & Oliva-Teles, 2001). Similarly, for blackspotted croaker a reduction of 7.5% of dietary protein level reduced ammonia excretion in about 20% (Li *et al.*, 2016a) and for red drum a reduction of dietary protein from 45 to 35% reduced ammonia excretion in about 22% (Webb & Gatlin, 2003) and reduced the 6h post-prandial ammonia production in about 56% (Wu & Gatlin, 2014). For the majority of the fish species lipids have proven to be more efficient energy suppliers than carbohydrates (Enes *et al.* 2010). Protein sparing by dietary lipids has been shown to occur in most fish species (Cho, 2008). However, magnitude of protein-sparing effect of lipid supplementation seems to be dependent dietary protein level, being more pronounced for lower protein levels (Dias *et al.* 1998). Indeed, sea bass fed diets including a protein level that meets protein requirements (Peres & Oliva-Teles, 1999b) no improvement of growth or protein utilization was achieved by increasing dietary lipid level from 12 to 24% (Peres and Oliva-Teles, 1999b). Similarly, for other fish species, it has also been reported that the increase of lipid level of diets with an adequate protein level as no major effect of protein utilization (McGoogan & Gatlin, 1999; Ozório *et al.* 2006; Velázquez *et al.* 2006; Bonaldo *et al.* 2010; Sevgili *et al.* 2014). In Senegalese sole, it was also observed that temperature may affect the degree of protein sparing by non-protein energy (Guerreiro *et al.* 2014). Some species, such as the Senegalese sole, are even less tolerant and high dietary lipid levels negatively affect growth and protein utilization (Dias *et al.* 2004; Guerreiro *et al.* 2012). The main concern of increasing dietary lipid level is the increase of lipid deposition in the carcass, which may negatively affect the nutritional value, organoleptic properties, transformation yield and storage time of fish. In fish, particularly in carnivorous fish, dietary carbohydrates utilization is limited (Stone, 2003; Enes *et al.* 2009; 2011). High dietary levels of carbohydrates lead to prolonged post-prandial hyperglycemia and inefficient carbohydrate utilization (Enes *et al.* 2011). Heat treatment may be required to improve digestive utilization of carbohydrates and modern technologies have allowed to extend the dietary incorporation of carbohydrates in diets of some fish species, like seabass and seabream, up to 20-30% (Couto *et al.* 2008; Oliva-Teles *et al.* 2011).

Table 2. Protein requirement of several marine fish species.

Species		Weight range (g)	Protein requirement (%)	Protein range tested (%)	Diet energy (MJ/GE)	Diet protein sources	References
Black Seabream	<i>Sparus macrocephalus</i>	13-56	41	32-49	16	FM	Zhang <i>et al.</i> 2010
Blackspot seabream	<i>Pagellus bogaraveo</i>	23-62	40	20-60	20	FM, pea meal, SBM	Silva <i>et al.</i> 2006
Blackspotted croaker	<i>Nibea diacanthus</i>	61-200	48.8	36-52	15.7	FM	Li <i>et al.</i> 2016a
Common dentex	<i>Dentex dentex</i>	2.5-15	50	40-55	21-22	FM, BM, SBM	Espinós <i>et al.</i> 2003
		10-37	50	50-57	22	FM, albumin	Skalli <i>et al.</i> 2004
		90-262	43	39-51	22	FM	Skalli <i>et al.</i> 2004
		17-25	49	44-59	21	FM, BM, SBM	Tibaldi <i>et al.</i> 1996
European seabass	<i>Dicentrarchus labrax</i>	5.6-30	48	36-56	20	FM	Peres & Oliva-Teles, 1999
		37-57	50	30-50	19	FM	Hidalgo & Alliot, 1988
Humpback grouper	<i>Cromileptes altivelis</i>	180-320	53	41-53	18-21	FM, MM, WM, casein	Usman <i>et al.</i> 2005
Hybrid grouper	<i>Epinephelus fuscoguttatus</i> × <i>E. lanceolatus</i>	2.5-64	50	40-55	18.8-21.6	FM, WM, DGM	Rahimnejad <i>et al.</i> 2015
Gilthead seabream	<i>Sparus aurata</i>	3-21	40	10-60		Casein, CPSP, AA	Sabaut & Luquet, 1973
		10-64	45	40-55	22	FM	Santinha <i>et al.</i> 1996
		0.8-3.3	55	35-65	20	FM	Vergara <i>et al.</i> 1996a
		5.5-30	46	42-58	20-21	FM	Vergara <i>et al.</i> 1996b
Malabar grouper	<i>Epinephelus malabaricus</i>	17-81	55	44-60	19-23.4	FM, krill, WG, casein	Tuan & Williams, 2007
Red spotted grouper	<i>Epinephelus akaara</i>	8-36	51	32-62	21	FM, casein	Wang <i>et al.</i> 2016a

Grouper	<i>Epinephelus coioides</i>	10-25	48	35-60	14	FM, casein	Luo <i>et al.</i> 2004
Japanese seabass	<i>Lateolabrax japonicas</i>	6.3-67.9	41	36-46	17.2	FM, SBM, BY, WM, SVM,	Ai <i>et al.</i> 2004

Table 2. (Continued).

Species		Weight range (g)	Protein requirement (%)	Protein range tested (%)	Diet energy (MJ/GE)	Diet protein sources	References
Olive flounder	<i>Paralichthys olivaceus</i>	256-442	45	40-50	18	FM	Kim <i>et al.</i> 2010
		4.1	51	35-65	17	FM	Kim <i>et al.</i> 2005
		13	44	30-60		FM	Kim <i>et al.</i> 2005
		8.1-40	45	25-60	17	FM	Kim <i>et al.</i> 2004
Patagonian blennie	<i>Eleginops maclovinus</i>	41-74	35	9-44	20	FM, WM	Sá <i>et al.</i> 2015
Red drum	<i>Sciaenops ocellatus</i>	2	40	35-45	14.5	Red drum muscle	Serrano <i>et al.</i> 1992

Table 2. (Continued).

Red seabream	<i>Pagrus major</i>	1.6-6.8	52	27-52		FM, casein	Takeuchi <i>et al.</i> 1991
Rockfish	<i>Sebastes schlegeli</i>	3.2-12.4	50	45-55	17.5-19	FM, casein, SBM	Cho <i>et al.</i> 2015
Senegalese sole	<i>Solea senegalensis</i>	6.4-24	50	45-50	19	FM; CG, WG	Guerreiro <i>et al.</i> 2012

		12-32	53	40-60	24	FM; CG, WG	
Sharpnout seabream	<i>Diplodus puntazzo</i>	49-113	52	14-68	21	FM	Coutinho <i>et al.</i> 2012
Turbot	<i>Scophthalmus maximus</i>	90-160	50	39-57	15	FM	Lee <i>et al.</i> 2003
Two-banded seabream	<i>Diplodus vulgaris</i>	6-20	36	5-55	22	FM	Ozório <i>et al.</i> 2009
White seabream	<i>Diplodus sargus</i>	22-36	27	6-49	21	FM	Sá <i>et al.</i> 2008
Yellowtail	<i>Seriola quinqueradiata</i>	3.6-33.6	50	35-55	(15% lipids)	FM	Takeuchi <i>et al.</i> 1992
Yellowtail kingfish	<i>Seriola lalandi</i>	>200	46		12	Commercial diet	Booth <i>et al.</i> 2010
		200-1000	47		15	Commercial diet	Booth <i>et al.</i> 2010
		>1000	43		18	Commercial diet	Booth <i>et al.</i> 2010
Zebra seabream	<i>Diplodus cervinus</i>	7-20	44	6-54	20-21	FM, CPSP	Coutinho <i>et al.</i> 2016a

BM, blood meal; BY, beer yeast; CG, corn gluten; CPSP, soluble fish protein concentrate; FM, fish meal; MM, mysid meal; SVM, squid visceral meal SBM, soybean meal; WG, Wheat gluten; Wheat meal, WM.

EAA Requirements

Ten essential amino acid (EAA) must be provided by dietary ingredients as they cannot be synthesized by fish (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine). Cysteine may be synthesized from methionine and tyrosine from phenylalanine, being considered semi-essential amino acids. Deficiency in only one of these AA will impair protein synthesis and depress fish growth (Wilson, 2002). Quantitative AA requirements for the 10 EAA were only determined for a few fish species (NRC, 2011).

For marine fish, quantitative requirements of some EAA was assessed by dose-response studies, based on animal growth or nitrogen retention responses to increasing dietary levels of the EAA under study (Table 3). Available data suggests some similarities but also a variation of EAA requirement among species. Part of this inter-species variation may be due to differences in methodological approaches, stressing the need for standardization methods (Dabrowski & Guderley, 2002). Several methodological issues have been raised including: the type and composition of the diet, which affect the capacity of fish to reach their maximum growth potential; the response criterion used to estimate requirements (growth, nitrogen retention, metabolic responses) and differences in the experimental design, statistical analysis and response modeling (Shearer, 2000; Bureau and Encarnaç o, 2006; Kaushik & Seiliez, 2010).

Table 3. Essential amino acids requirements (g/16g N) of some marine fish species estimated by dose-response studies.

	Arg	Lys	Met	Thr	Phe	Try	Tau	References
Asian seabass, <i>Lates calcarifer</i>	3.8	4.5	2.2					Murillo Gurrea <i>et al.</i> 2001; Coloso <i>et al.</i> 1999, 2004
Atlantic salmon, <i>Salmo salar</i>	4.1-5.1							Berge <i>et al.</i> 1997; Lall <i>et al.</i> 1994
Black sea bream, <i>Sparus macrocephalus</i>	7.7-8.1	8.6	4.5					Zhou <i>et al.</i> 2010a,b, 2011
Cobia, <i>Rachycentron canadum</i>	6.2	5.3	2.2-2.6					Zhou <i>et al.</i> 2006, 2007; Ren <i>et al.</i> 2014; Wang <i>et al.</i> 2016b
European seabass, <i>Dicentrarchus labrax</i>	3.9-4.6	4.82	0.9-2.7 (1.31)	2.3-2.6		0.5-0.7		Thebault <i>et al.</i> 1985; Tibaldi and Lanari, 1991; Tibaldi <i>et al.</i> 1993a,b, 1994; Tibaldi and Tulli, 1999; Tulli <i>et al.</i> 2010
Gilthead seabream, <i>Sparus aurata</i>	<2.6	5.0	2.8 (4.0)			0.6		Luquet and Sabaut, 1974; Marcouli <i>et al.</i> 2006
Golden pompano, <i>Trachinotus ovatus</i>	6.3		2.5-3					Niu <i>et al.</i> 2013; Lin <i>et al.</i> 201
Grouper, <i>Epinephelus coioides</i>		5.6	2.73					Luo <i>et al.</i> , 2005, 2006
Japanese flounder, <i>Paralichthys olivaceus</i>	2.1	4.2-4.6	2.9	3.2				Forster and Ogata, 1998, Alam <i>et al.</i> 2000, 2002; 2003
Japanese seabass, <i>Lateolabrax japonicas</i>		5.8-6.1						Mai <i>et al.</i> 2006
Milkfish, <i>Chanos chanos</i>	5.3	4		4.5		0.6		Borlongan, 1991; Borlongan and Benitez, 1990; Coloso <i>et al.</i> 1992

Pacific threadfin, <i>Polydactylus sexfilis</i>		5.1				-	Deng <i>et al.</i> 2010
Red drum, <i>Sciaenops ocellatus</i>	4.2-5.0	4.4-5.7	3.0	2.3	1.69 (0.41 Tyr)	0.8	Brown <i>et al.</i> 1988; Moon and Gatlin, 1991; Craig and Gatling, 1992; Boren and Gatling, 1995; Barziza <i>et al.</i> 2000; Castillo <i>et al.</i> 2015; Pewitt <i>et al.</i> 2017
Red seabream, <i>Pagrus major</i>	4.7	3.6-4.4					Forster and Ogata, 1998; Rahimnejad and Lee., 2014
Rockfish, <i>Sebastes schlegeli</i>			2.8				Yan <i>et al.</i> 2007
Striped Bass, <i>Morone saxatilis</i>	4.1-4.4	4.3-4.9		2.45		0.6-0.7	Keembiyehetty <i>et al.</i> 1992; Griffin <i>et al.</i> 1994; Small, <i>et al.</i> 1999, 2000; Gaylord <i>et al.</i> 2005
Turbot, <i>Scophthalmus maximus</i>		5	2.6-3.3			0.9-1.9	Peres and Oliva-Teles, 2008; Qi <i>et al.</i> 2012; Ma <i>et al.</i> 2013; Klatt <i>et al.</i> 2016
Yellow croaker <i>Pseudosciaena crocea</i>		5.7-6.6	3.2-3.3				Mai <i>et al.</i> 2006; Zhang <i>et al.</i> 2008; Xie <i>et al.</i> 2012; Li <i>et al.</i> 2014
Yellow grouper <i>Epinephelus awoara</i>	6.5						Zhou <i>et al.</i> 2012
Yellowtail, <i>Seriola quinqueradiata</i>	3.4-3.8	4.1	3.3				Ruchimat <i>et al.</i> 1997a, b; Ruchimat <i>et al.</i> 1998

Special emphasis should be given to the composition of experimental diets (Nunes *et al.* 2014). In dose-response studies, purified or semi-purified diets are often used, in which protein bound-AA are replaced by crystalline-AA. In some studies, use of such diets was proven to be less effective in promoting fish growth than diets containing only intact protein (Cowey, 1995; Zarate and Lovell, 1997). This may be an issue, as it is important that fish fed the semi-purified diets perform as well as fish fed practical diets. Thus, for an unbiased evaluation of EAA requirement it is necessary to confirm that crystalline-AA are effectively utilized by the animals (Peres & Oliva-Teles, 2005). Maximum tolerable levels of crystalline-AA that do not impair growth performance or feed utilization efficiency may depend on fish species. For instance, in turbot a maximum of 19% of protein replacement by free AA was achieved without affecting growth performance (Peres & Oliva-Teles, 2005) but higher inclusion levels, around 30-55% of dietary protein, were reported as feasible for other species, such as gilthead seabream, red seabream, European seabass or Senegalese sole (Webb & Gatlin, 2003; Marcouli *et al.* 2005; Alam *et al.* 2005; Peres and Oliva-Teles, 2007; Perez-Jimenez *et al.* 2014).

Dietary EAA profile, EAA to non-EAA ratio and composition of the non-EAA fraction of the diets are also important issues, as it affects growth and protein utilization (Mambrini and Kaushik, 1995; Kaushik & Seiliez, 2010). The whole-body EAA profile is usually used as the most appropriate EAA profile for the ideal protein, however for marine species such as gilthead seabream, red seabream and European seabass, the fish meal EAA profile also provides satisfactory results (Marcouli *et al.* 2005; Alam *et al.* 2005; Peres & Oliva-Teles, 2007; Fig. 1). This is not unexpected, as fish meal is usually considered the most adequate protein source for fish and the EAA profile of fish species is not very different.

To ensure best performances and feed utilization, an adequate amount of non-EAA should be provided in the diets, as EAA are not always efficiently used as non-specific nitrogen source for synthesis of non-EAA. In fact, diets with low levels of non-EAA are used less efficiently than diets with adequate EAA to non-EAA ratios in chickens, pigs and fish (Mambrini and Kaushik, 1994; Schuhmacher *et al.* 1995; Abboudi *et al.* 2009). For instance, the most adequate dietary EAA to non-EAA ratio for seabass was estimated to be 0.55-0.60 (Peres & Oliva-Teles, 2006; Fig. 1), which is similar to the ratios established for

salmonids and terrestrial animals (Green *et al.* 2002; Gomez-Requeni *et al.* 2003). Another important issue when using diets with protein-bound and crystalline-AA is to assure that the absorption of crystalline-AA is synchronized with that of protein bound-AA, for guaranteeing that all AA are present in the body AA-pool simultaneously (Fournier *et al.* 2002; Peres & Oliva-Teles, 2005, 2006, 2007). Otherwise, an unbalanced AA availability may occur at physiological and metabolic levels. Thus, it may be required to consider pre-coating crystalline-AA with agar or gelatin to delay their absorption. Besides, coating crystalline-AA also reduces leaching of crystalline-AA during diet exposure to water, and avoids problems related to the acidic diet in stomachless fish.

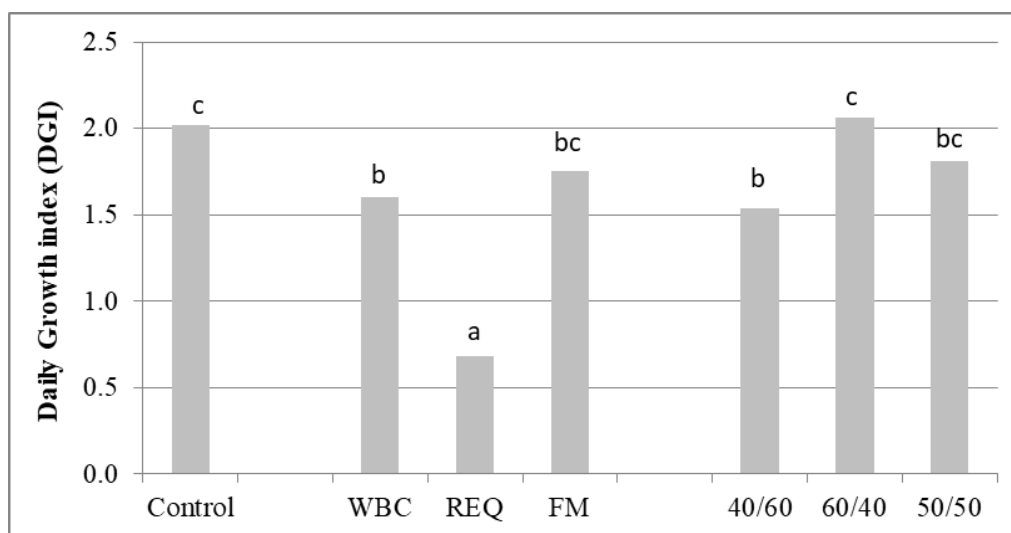


Fig 1. Comparison of European seabass performance fed a control diet (containing only intact protein) and test diets (containing free-AA replacing 60% of intact protein nitrogen of control diet) with different EAA profile and EAA/non-EAA ratio. WBC: whole body EAA profile; REQ: ideal protein EAA profile; FM: fish meal EAA profile) (Data from Peres & Oliva-Teles, 2006; 2007).

Despite the variability of EAA requirements among species, some similar pattern is also evident (Table 3). Hauler and Carter (2001) compiled available data on lysine requirement of fish species and concluded that in spite of the high variance of lysine requirements, requirements per unit of body mass gain was relatively stable, being estimated to be 18.5 g lysine for kg of whole-body weight. Kaushik and Seiliez (2010)

using a meta-analysis of the available data on lysine and methionine requirement in salmonids were able to draw similar dose-response patterns independent of species.

For marine fish species, meta-analysis of available data on lysine, methionine and arginine requirement for marine fish is presented in Fig 2, 3 and 4. For the other EAA, due to the limited number study, such approach was not possible. The four-parameter saturation kinetic model (Mercer *et al.* 1984) was used to analyze dose-response data. Experimental condition, growth rate and initial body weight greatly differ among studies, and therefore it was necessary to standardize the response criterion relatively to the maximum weight gain or maximum thermal growth coefficient within each study. This standardization is necessary for a direct interstudy comparison of growth data, due to differences in fish size, trail duration and magnitude of somatic growth in absolute terms. For lysine, methionine and arginine a compilation of 17, 11 and 16 studies, respectively, was used (see table 4, 5 and 6, respectively for references). Despite the scarcity of studies on EAA requirements for marine fish, data used for this meta-analysis fitted relatively well with the model used. Based on this meta-analysis, dietary lysine, methionine and arginine requirement to meet 95% of maximum weight gain of fish were estimated to be: 4.90 and 5.42 (g/16g N) for lysine (Fig. 2); 2.41 and 2.74 (g/16g N) for methionine (Fig. 3) and 4.54 and 4.73 (g/16g N) for arginine (Fig. 4).

The meta-analysis values obtained for lysine and methionine requirements fit well with the individual dose-response requirements evaluation (ranging from 3.3 to 56.5 and from 2.3 to 4.5 g/16g N for lysine and methionine, respectively; Table 4 and 5). Arginine requirement estimated by the meta-analysis was also within the values obtained based on dose-response studies (ranging from 3.8 to 8.1 g/16g N; Table 6), but although high, the R value explaining the fitness of arginine data was lower than that obtained for the other two amino acids. This discrepancy may be related to the range of values used in the dose-response studies used for fitting the meta-analysis models. Thus, more data is required for a better adjustment of the model and to have more confidence on the results (Fig 5).

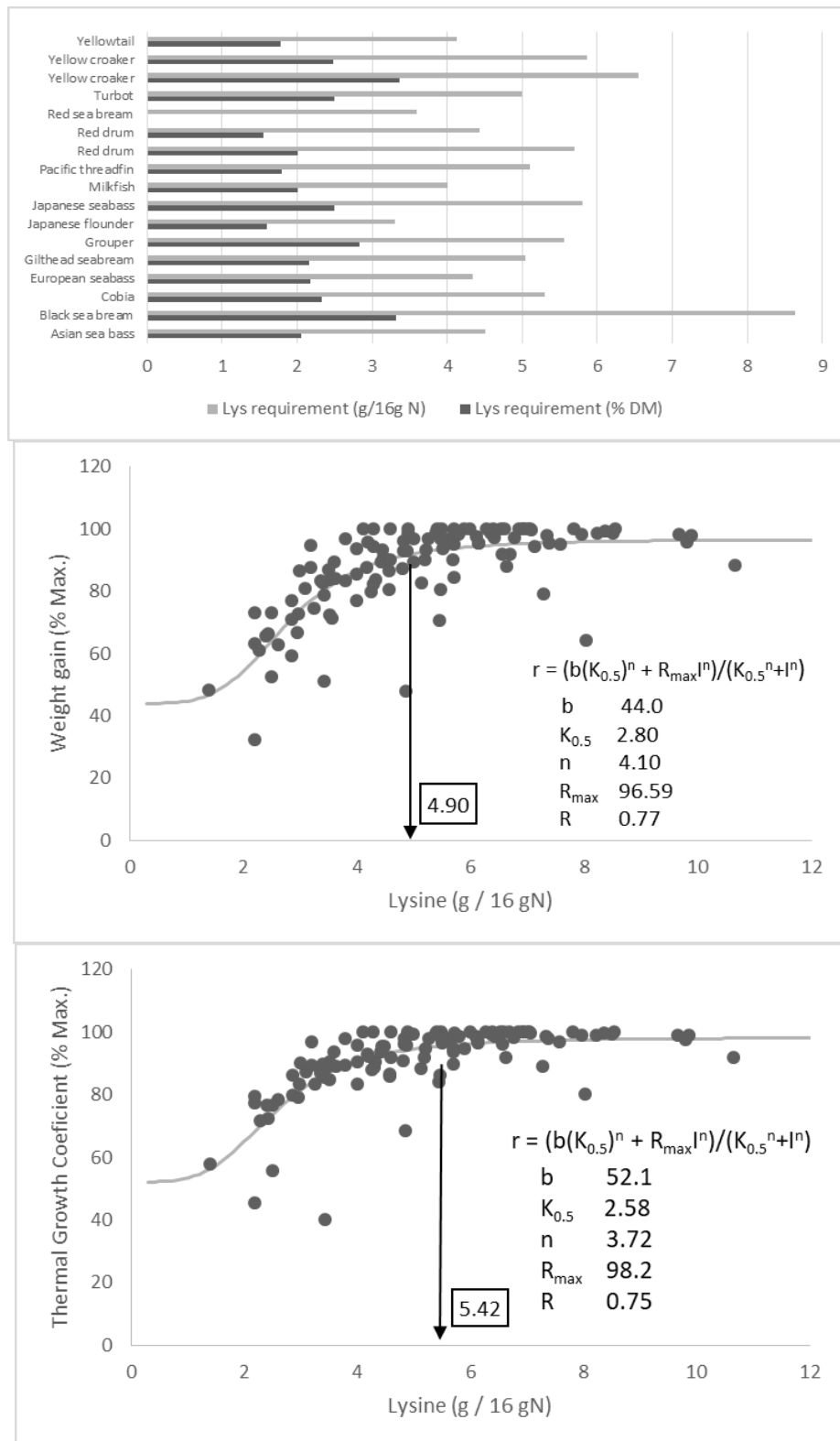
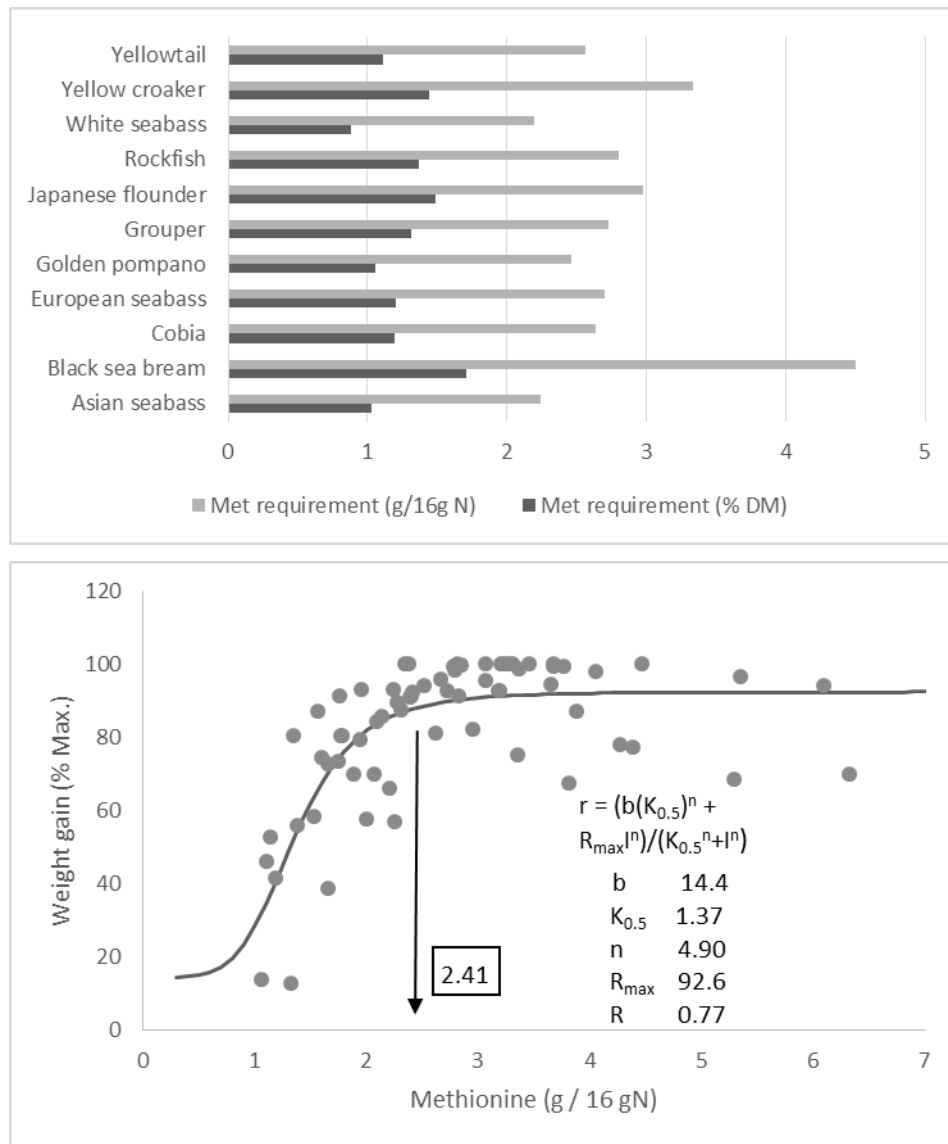


Fig 2. Analysis of literature data on lysine requirements of marine fish species (references in Table 4).



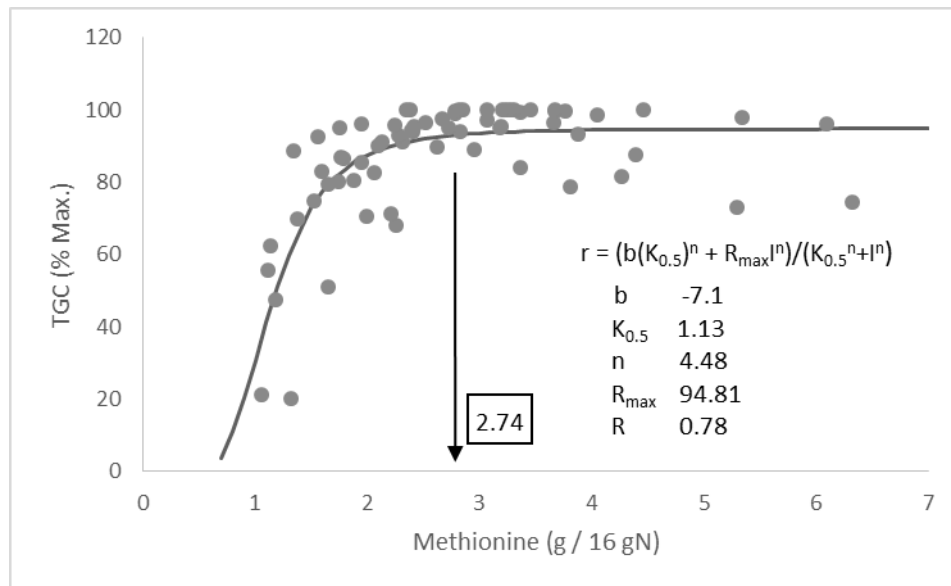


Fig 3. Analysis of literature data on methionine requirements of marine fish species (references in Table 5).

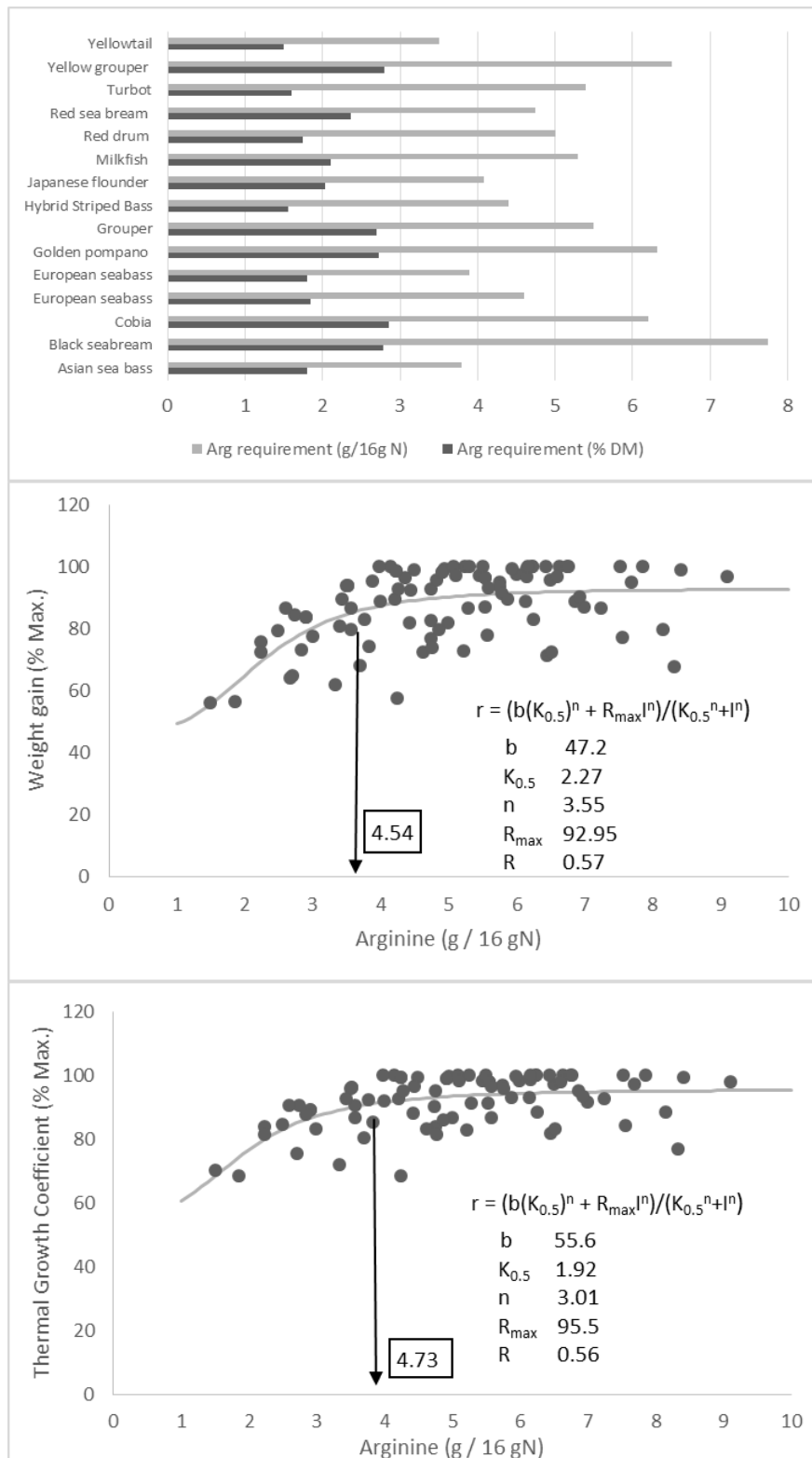


Fig 4. Analysis of literature data on arginine requirements of marine fish species (references in Table 6).

Table 4. Data collected from dose-response lysine (Lys) requirement studies.

Common name	Scientific name	Initial body weight (g/fish)	Water temperature (°C)	Trial duration (days)	Feeding Regimen	Protein ingredients	Diet crude protein (%)	Diet crude lipid (%)	Energy (kJ/g)	References
Asian sea bass	<i>Lates calcarifer</i>	13.1	27	84	FR	FM, zein, SM	45.5	10	-	Murillo-Gurrea <i>et al.</i> 2001
Black sea bream	<i>Sparus macrocephalus</i>	9.1	28	56	Satiation	FM, SPC, Gel	38	15	15.7(DE)	Zhou <i>et al.</i> 2010b
Cobia	<i>Rachycentron canadum</i>	1.25	28.5	56	Satiation	FM, WG	44	16	-	Zhou <i>et al.</i> 2007b
European seabass	<i>Dicentrarchus labrax</i>	0.85	25.5	70	FR	MG, HM, Gel	50	14	17.5(DE)	Tibaldi & Lanari, 1991
Gilthead seabream	<i>Sparus aurata</i>	3.5	24	42	Satiation	LT-FM	43	13.5	-	Marcouli <i>et al.</i> 2006
Grouper	<i>Epinephelus coioides</i>	15.8	29	56	Satiation	FM, SPC	48	10	-	Luo <i>et al.</i> 2006
Hybrid striped bass	<i>(Morone chrysops × saxatilis)</i>	8	24.5	63	FR	Red drum muscle,	48	10	15	Keembiyehetty and Gatlin, 1992
Hybrid striped bass	<i>(Morone chrysops × saxatilis)</i>	11	28	56	FR	Casein, Gel	35			Griffin <i>et al.</i> 1992
Japanese flounder	<i>Paralichthys olivaceus</i>	3	19.5	63	Satiation	Zein, FM, Gel	47	11	-	Forster & Ogata, 1998
Japanese seabass	<i>Lateolabrax japonicus</i>	5.5	28.5	70	Satiation	FM, SBM, zein, WM	43	12	-	Mai <i>et al.</i> 2006
Milkfish	<i>Chanos chanos</i>	5.92	29	84	FR	FM, zein	50	-	-	Borlongan & Benitez, 1990
Pacific threadfin	<i>Polydactylus sexfilis</i>	3	25	56	Satiation	Pa. threadfin muscle	35	11.5	19.1(GE)	Deng <i>et al.</i> 2010
Red drum	<i>Sciaenops ocellatus</i>	2.7	28	56	FR	PM, ShM	35	-	-	Brown <i>et al.</i> 1988

Red drum	<i>Sciaenops ocellatus</i>	6-7/1-2	-	56	FR	Red drum muscle	35	-	-	Craig and Gatlin, 1992
Red sea bream	<i>Pagrus major</i>	1.7	22.5	56	Satiation	Zein, FM, Gel	48	14	-	Forster & Ogata, 1998
Turbot	<i>Scophthalmus maximus</i>	18.1	18	56	Satiation	MG, FM, SFPC	50	15	18.5(DE)	Peres & Oliva-Teles, 2008
Yellow croaker	<i>Pseudosciaena crocea</i>	0.03	23	30	Satiation	FM, KM, SM, MG	51	18.5	22.3	Xie <i>et al.</i> 2012
Yellow croaker	<i>Pseudosciaena crocea</i>	1.23	28.5	70	Satiation	FM, SBM, CGM, WM	43	12	-	Zhang <i>et al.</i> 2008
Yellowtail	<i>Seriola quinqueradiata</i>	68.2	25.8	40	Satiation	FM, MG, WG	43.1	15.9	15.5(DE)	Ruchimat <i>et al.</i> 1997a

Table 4. (Continued).

Common name	Scientific name	Lys levels (% diet)	Lys levels (g/16gN)	TGC	Nitrogen retention (%NI)	Lys requirem. (% DM)	Lys requirem. (g/16g N)	Response variable	Statistical Model	References
Asian sea bass	<i>Lates calcarifer</i>	1.0-2.5	2.17-5.43	1.56-1.97		2.06	4.5	WG/FER	BL	Murillo Gurrea <i>et al.</i> 2001
Black sea bream	<i>Sparus macrocephalus</i>	2.08-4.05	5.5-10.7	0.7-0.81	28-34	3.32	8.64	SGR	SOPR	Zhou <i>et al.</i> 2010b
Cobia	<i>Rachycentron canadum</i>	1.15-3.25	2.6-7.4	0.90-1.13		2.33	5.3	SGR	BL	Zhou <i>et al.</i> 2007
European seabass	<i>Dicentrarchus labrax</i>	1.20-2.45	2.4-4.9	0.36-0.47	14.9-26.1	2.17/2.22	4.34/4.44	WG/GPR	BL	Tibaldi & Lanari, 1991
Gilthead seabream	<i>Sparus aurata</i>	1.56-3.43	3.63-7.97	0.84-0.95	34.4-39.9	2.16	5.04	DPD	SKM	Marcouli <i>et al.</i> 2006
Grouper	<i>Epinephelus coioides</i>	1.92-3.95	4-8.2	0.65-0.91	24-30	2.83	5.56	WG	BL	Luo <i>et al.</i> 2006
Japanese flounder	<i>Paralichthys olivaceus</i>	1.03-3.1	2.2-6.6	0.40-0.88	8.2-20.5	1.6/2.0/2.2	3.3/4.2/4.6	SGR/FE/NR	BL	Forster and Ogata, 1998
Japanese seabass	<i>Lateolabrax japonicus</i>	1.28-4.25	3-9.9	0.83-1.00		2.49/2.61/2.6	5.8/6.07/6.05	SGR/FER/PER	BL	Mai <i>et al.</i> 2006
Milkfish	<i>Chanos chanos</i>	0.7-2.7	1.4-5.4	0.2-0.35		2	4	WG	SOPR	Borlongan & Benitez, 1990
Pacific threadfin	<i>Polydactylus sexfilis</i>	1.26-2.91	3.6-8.31	1.11-1.18	34.6-40.3	1.79	5.1	SGR	BL	Deng <i>et al.</i> 2010
Red drum	<i>Sciaenops ocellatus</i>	1.2-2.4	3.4-6.9	0.18-0.44		2	5.7	SFLC	MRT	Brown <i>et al.</i> 1988
Red drum	<i>Sciaenops ocellatus</i>	1.0-2.5	2.9-7.1	1.49-2.14 (DGI)		1.55	4.43	WG, FE	BL	Craig and Gatlin, 1992
Red sea bream	<i>Pagrus major</i>	1.23-3.16	2.5-6.7	0.65-0.97	27.4-37.9	1.7/2.1/2.1	3.6/4.3/4.4	SGR/FE/NR	BL	Forster & Ogata, 1998
Turbot	<i>Scophthalmus</i>	1.19-3.11	2.5-6.4	0.45-0.58	20.4-30	2.5	5.0	WG	SKM	Peres & Oliva-Teles,

Peres, H. and Oliva, A. 2017. Protein and amino acid nutrition of marine fish species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. (Eds), Investigación y Desarrollo en Nutrición Acuícola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 438-492. ISBN 978-607-27-0822-8.

	<i>maximus</i>								2008	
Yellow croaker	<i>Pseudosciaena</i>	2.48-4.10	4.86-8.03	0.25-0.36		3.37-3-34	6.55-6.49	SGR/survival	SOPR	Xie <i>et al.</i> 2012
	<i>crocea</i>									
Yellow croaker	<i>Pseudosciaena</i>	1.27-4.22	2.95-9.81	0.48-0.61		2.48/2.45/2.43	5.87/5.70/5.65	SGR/FE/PER	BL	Zhang <i>et al.</i> 2008
	<i>crocea</i>									
Yellowtail	<i>Seriola</i>	1.05-3.05	2.4-7.1	0.80-0.95	15.6-22.6	1.78	4.13	WG, FE	BL	Ruchimat <i>et al.</i> 1997a
	<i>quinqueradiata</i>									

ANOVA, analysis of variance; ANU, apparent nitrogen utilization; BL, broken line regression; DE, digestible energy; DGI, daily growth index; DPD, daily protein deposition; EF, exponential function; FCR, feed conversion ratio; FE, feed efficiency; FER, feed efficiency ratio; FM, fish meal; FR: Fixed ration; GE, gross energy; Gel, gelatin; GPR, gross protein retention; HM, herring meal; MG, maize gluten; MRT, Duncan's multiple-rang test; NR, nitrogen retention; PD, protein deposition; PER, protein efficiency ratio; PM, peanut meal; PRE, protein retention efficiency; RGM, rice gluten meal; SBM, soy-bean meal; SFLC, serum free lysine concentration; SFPC, soluble fish protein concentrate; SGR, specific growth rate; SGR, specific growth rate; ShM, shrimp meal; SKM-4, saturation kinetic model-4 parameter; SM, squid meal; SOPR, second order polynomial regression; SPC, soy-bean protein concentrate; TGC: thermal growth coefficient WG, weight gain; WG, wheat gluten.

Table 5. Data collected from dose-response methionine (Met) requirement studies.

Common name	Scientific name	Initial body weight (g/fish)	Water temperature (°C)	Trial duration (days)	Feeding Regimen	Protein ingredients	Diet crude protein (%)	Diet crude lipid (%)	Energy (kJ/g)	References
Asian seabass	<i>Lates calcarifer</i>	2.59	26	84	FR	FM, SM, SBM	46	10.5	16	Coloso <i>et al.</i> 1999
Black sea bream	<i>Sparus macrocephalus</i>	14.21	28	56	Satiation	FM, SPC	38.5	15	15.7	Zhou <i>et al.</i> 2011
Cobia	<i>Rachycentron canadum</i>	11.61	29	56	Satiation	Brown FM, WG	44	16		Zhou <i>et al.</i> 2006
European seabass	<i>Dicentrarchus labrax</i>	13.4	23	84	Satiation	FPC, WG, Gel, SPC, SBM	44	14.5	19 (GE)	Tulli <i>et al.</i> 2010
Golden pompano	<i>Trachinotus ovatus</i>	12.4	29.5	56	Satiation	FM, MG, SBM, PM, SPC	43	13		Niu <i>et al.</i> 2013
Grouper	<i>Epinephelus coioides</i>	13.25	29.25	56	Satiation	FM, SPC	48	9.8	14.3 (DE)	Luo <i>et al.</i> 2005
Japanese flounder	<i>Paralichthys olivaceus</i>	2.8	21.6	40	FR	Casein, Gel	50	9.6		Alam <i>et al.</i> 2000
Rockfish	<i>Sebastes schlegeli</i>	43.61	19.5	65	Satiation	FM	48.7	10.4	18.9	Yan <i>et al.</i> 2007
White seabass	<i>Atractoscion nobilis</i>	5.6	18	57	FR	FM, SBM; SPC, CGM, WM	40	10		Salze <i>et al.</i> 2017
Yellow croaker	<i>Pseudosciaena crocea</i>	1.23	28.5	70	Satiation	FM, SBM, WM	43	12		Mai <i>et al.</i> 2006
Yellowtail	<i>Seriola quinqueradiata</i>	23.3	24.8	30	Satiation	FM, SPC, WG, Gel	43	17	16.3 (DE)	Ruchimat <i>et al.</i> 1997b

Table 5. (Continued).

Common name	Scientific name	Met levels (% diet)	Met levels (g/16gN)	Cys level (% diet)	TGC	Nitrogen retention (%NI)	Met requirement (% DM)	Met requirement (g/16g N)	Response variable	Statistical Model	References
Asian seabass	<i>Lates calcarifer</i>	0.62-1.08	1.35-2.35	0.31	0.69-0.77		1.03	2.24	WG	BL	Coloso <i>et al.</i> 1999
Black sea bream	<i>Sparus macrocephalus</i>	0.75-2.35	1.9-6.1	0.31	0.84-0.98	30-43	1.71 / 1.72	4.5	SGR/PPV	SOPR	Zhou <i>et al.</i> 2011
Cobia	<i>Rachycentron canadum</i>	0.61-1.68	1.4-3.8	0.67	0.92-1.32		1.19	2.64	SGR	QRA	Zhou <i>et al.</i> 2006
European seabass	<i>Dicentrarchus labrax</i>	0.49-1.62	1.1-3.7	0.4	0.32-0.58	15.69- 27.52	1.2/0.8	2.7/1.8	WG/PD	BL	Tulli <i>et al.</i> , 2010
Golden pompano	<i>Trachinotus ovatus</i>	0.86-1.45	2-3.4	0.2	0.86-1.2	15.7-24.1	1.06-1.27	2.46-2.95	WG, NRE	BL	Niu <i>et al.</i> 2013
Grouper	<i>Epinephelus coioides</i>	0.55-1.81	1.1-3.8	0.26	0.47-0.76	16.3-31.6	1.31	2.73	WG	BL	Luo <i>et al.</i> 2005
Japanese flounder	<i>Paralichthys olivaceus</i>	0.53-2.03	1.06-4.06	0.06	0.22-1.05	7.3-25.8	1.49 / 1.44	2.98 / 2.88	WG / FE	BL	Alam <i>et al.</i> 2000
Rockfish	<i>Sebastes schlegeli</i>	0.58-3.08	1.2-6.3	0.12	0.34-0.72	18.1-30.8	1.37	2.8	SGR	ANOVA	Yan <i>et al.</i> 2007
White seabass	<i>Atractoscion nobilis</i>	0.64-1.28	1.6-3.2	0.51	1.11-1.35		0.88-1.21	2.22-3.00	TGC	SKM/ BQM	Salze <i>et al.</i> 2017
Yellow croaker	<i>Pseudosciaena crocea</i>	0.66-1.89	1.5-4.4	0.29	0.61-0.81		1.44 / 1.39	3.34 / 3.22	SGR/FCE	SOPR	Mai <i>et al.</i> 2006
Yellowtail	<i>Seriola quinqueradiata</i>	0.57-1.58	1.3-3.7	0.31	0.52-2.59	4.2-40.8	1.11	2.56	WG, FE	BL	Ruchimat <i>et al.</i> 1997b

ANOVA, analysis of variance; ANU, apparent nitrogen utilization; BL, broken line regression; BQM, broken quadratic model; DE, digestible energy; DGI, daily growth index; DPD, daily protein deposition; EF, exponential function; FCR, feed conversion ratio; FE, feed efficiency; FER, feed efficiency ratio; FM, fish meal; FR: Fixed ration; GE, gross energy; Gel, gelatin; GPR, gross protein retention; HM, herring meal; MG, maize gluten; MRT, Duncan's multiple-rang test; NR, nitrogen retention; PD, protein deposition; PER, protein efficiency ratio; PM, peanut meal; PRE, protein retention efficiency; RGM, rice gluten meal; SBM, soy-bean meal; SFLC, serum free lysine concentration; SFPC, soluble fish protein concentrate; SGR, specific growth rate; SGR, specific growth rate; ShM, shrimp meal; SKM-4, saturation kinetic model-4 parameter; SM, squid meal; SOPR, second order polynomial regression; SPC, soy-bean protein concentrate; TGC: thermal growth coefficient WG, weight gain; WG, wheat gluten.

Table 6. Data collected from dose-response arginine (Arg) requirement studies.

Common name	Scientific name	Initial body weight (g/fish)	Water temperature (°C)	Trial duration (days)	Feeding Regimen	Protein ingredients	Diet crude protein (%)	Diet crude lipid (%)	Energy (kJ/g)	References
Asian sea bass	<i>Lates calcarifer</i>	2.6	29	85	FR	FM, zein, SM	48.5	10.3		Murillo-Gurrea <i>et al.</i> 2001
Black seabream	<i>Sparus macrocephalus</i>	10.5	28	56	Satiation	FM, SPC	38	14.2	15.5	Zhou <i>et al.</i> 2010c
Cobia	<i>Rachycentron canadum</i>	3.4	30.5	63	Satiation	FM, CGM	46	14		Ren <i>et al.</i> 2014
European seabass	<i>Dicentrarchus labrax</i>	0.8	26	60	Satiation	HM, CGM	40	12.6		Tibaldi <i>et al.</i> 1993
European seabass	<i>Dicentrarchus labrax</i>	2.1	25	63	FR	MG, HM	46	12		Tibaldi <i>et al.</i> 1994
Golden pompano	<i>Trachinotus ovatus</i>	18.8	25	56	Satiation	FM, CGM, RSM, PNM	43	12.5		Lin <i>et al.</i> 2015
Grouper	<i>Epinephelus coioides</i>	16	26.5	56	Satiation	FM, SPC	48	11	17.3	Luo <i>et al.</i> 2007
Hybrid Striped Bass	<i>M.saxatilis x M.chrysops</i>	7.2	28	70	Satiation	Casein, Gel	35	6	19.2	Griffin <i>et al.</i> 1994
Hybrid Striped Bass	<i>M.saxatilis x .chrysops</i>	3.1								Griffin <i>et al.</i> 1994
Japanese flounder	<i>Paralichthys olivaceus</i>	1.9	21	40	FR	Casein, Gel	50	9.8		Alam <i>et al.</i> 2002
Milkfish	<i>Chanos chanos</i>	0.7	29	84	Satiation	Casein, Gel	40			Borlongan, 1991
Red drum	<i>Sciaenops ocellatus</i>	3.8	29	49	FR	Red drum muscle	35		13.4	Barziza <i>et al.</i> 2000
Red sea bream	<i>Pagrus major</i>	13.3		63	Satiation	FM, SPC	50	15		Rahimnejad & Lee, 2014
Turbot	<i>Psetta maxima</i>	7.4	17	84	Satiation	FM, FPC, CGM	56	18	24	Fournier <i>et al.</i> 2003
Yellow grouper	<i>Epinephelus awoara</i>	4.2	28	56	Satiation	FM, SBM	43	10		Zhou <i>et al.</i> 2012b
Yellowtail	<i>Seriola quinqueradiata</i>	32.0		28	Satiation	Casein, FM	42		15.3	Ruchimat <i>et al.</i> 1998

Table 6. (Continued).

Common name	Scientific name	Arg levels (% diet)	Arg levels (g/16gN)	Thermal growth coefficient	Nitrogen retention (%NI)	Arg requirement (% DM)	Arg requirement (g/16gN)	Response variable	Statistical Model	Reference
Asian sea bass	<i>Lates calcarifer</i>	0.73-3.73	1.59-8.11	0.39-0.56		1.8	3.8	WG/FER	BL	Murillo Gurrea <i>et al.</i> 2001
Black seabream	<i>Sparus macrocephalus</i>	1.85-3.46	4.9-9.1	0.83-0.97	36-44	2.79/3.1	7.74/8.1	SGR/PER	BL/SOPR	Zhou <i>et al.</i> 2010b
Cobia	<i>Rachycentron canadum</i>	1.76-3.75	3.8-8.2	0.90-1.06		2.85	6.2	WG	SOPR	Ren <i>et al.</i> 2014
European seabass	<i>Dicentrarchus labrax</i>	1.07-2.70	2.68-5.19	0.53-0.69		1.85	4.6	WG	BL	Tibaldi <i>et al.</i> 1993
European seabass	<i>Dicentrarchus labrax</i>	1.8	2.2-6.2	0.53-0.63	17-22	1.8	3.9	WG	BL/SKM	Tibaldi <i>et al.</i> 1994
Golden pompano	<i>Trachinotus ovatus</i>	2.05-3.58	4.8-8.3	1-1.23		2.73	6.32	WG	QRA	Lin <i>et al.</i> 2015
Grouper	<i>Epinephelus coioides</i>	1.78-3.31	3.7-6.9	0.97-0.96		2.7	5.5	WG	BL	Luo <i>et al.</i> 2007
Hybrid	<i>M.saxatilis x</i>	1-2.4	2.5-6.0	0.39-0.47		1.55	4.4	WG	BL	Griffin <i>et al.</i> 1994
Striped Bass	<i>M.chrysops</i>									
Japanese flounder	<i>Paralichthys olivaceus</i>	2.0	2.5-6.5	0.46-0.92	19-48	2.04/2.1	4.08 / 4.2	WG/FCE	BL	Alam <i>et al.</i> 2002
Milkfish	<i>Chanos chanos</i>	2.1	3.65-6.25	0.13-0.24		2.1	5.3	WG	BL	Borlongan, 1991
Red drum	<i>Sciaenops ocellatus</i>	1.8	1.9-7.9	0.57-0.84	22-29	1.75/1.44/1.48	5/4.11/4.23	WG/FE/PER	BL	Barziza <i>et al.</i> 2000
Red sea bream	<i>Pagrus major</i>	1.42-3.08	2.8-6.2	0.85-0.97		2.37	4.74	WG	BL	Rahimnejad & Lee, 2014
Turbot	<i>Psetta maxima</i>					1.6-3	3-5.4	WG	ANOVA	Fournier <i>et al.</i> 2003
Yellow grouper	<i>Epinephelus awoara</i>	2.8	4.7-7.6	0.63-0.73	20-23	2.8	6.5	SGR, WG	QRA	Zhou <i>et al.</i> 2012b
Yellowtail	<i>Seriola quinqueradiata</i>	1.5	3.4-5.8	5.44-6.36 (DGI)	21-27	1.5	3.5	WG	MRT	Ruchimat <i>et al.</i> 1998

ANOVA, analysis of variance; ANU, apparent nitrogen utilization; BL, broken line regression; DE, digestible energy; DGI, daily growth index; DPD, daily protein deposition; EF, exponential function; FCR, feed conversion ratio; FE, feed efficiency; FER, feed efficiency ratio; FM, fish meal; FR: Fixed ration; GE, gross energy; Gel, gelatin; GPR, gross protein retention; HM, herring meal; MG, maize gluten; MRT, Duncan's multiple-rang test; NR, nitrogen retention; PD, protein deposition; PER, protein efficiency ratio; PM, peanut meal; PNM: peanut meal; PRE, protein retention efficiency; RSM, Rapeseed meal, RGM, rice gluten meal; SBM, soy-bean meal; SFLC, serum free lysine concentration; SFPC, soluble fish protein concentrate; SGR, specific growth rate; SGR, specific growth rate; ShM, shrimp meal; SKM-4, saturation kinetic model-4 parameter; SM, squid meal; SOPR, second order polynomial regression; SPC, soy-bean protein concentrate; WG, weight gain; WG, wheat gluten.

Ideal Protein

The concept of ideal protein is that for a species, in a given physiological stage, there is an optimum dietary EAA profile that meets maintenance and production needs and maximizes nitrogen retention (Boisen, 2003). A protein with this optimum EAA profile is considered an “ideal protein”. In this ideal protein, each EAA can be computed as a proportion of the total EAA amount ($A/E \text{ ratios} = \text{EAA content} / \text{total EAA content including cysteine and tyrosine}$). Thus, quantitative requirements of all EAA can be estimated based on the A/E ratio, provided that the requirement of a single EAA is known. The EAA requirements of all other EAA is then expressed as a proportion of the reference EAA according to the relative proportion of each one in the optimum EAA profile.

Estimation of the EAA requirements based on the ideal protein concept is as a highly versatile approach that is being used extensively in pigs (Boisen, 2003) and poultry (Baker, 2003). This approach provides reliable and fast information of the EAA requirements, and is more economical than the classical dose-response method, and allows that modifications in requirements for all EAA related to animal age/size, physiological or environmental conditions can be easily estimated by evaluating changes in the requirement of a single amino acid. For consistent results with this approach it is required that reliable information on the optimum EAA profile for the animal is obtained and that the requirement of the EAA used as reference is accurately established (Boisen, 2003).

Lysine has been used as the reference AA in almost studies using the ideal protein approach, because it this AA is used mostly for protein accretion; it lacks metabolic interactions with other AA; its requirements is usually the highest among EAA; and it is generally the first limiting AA in practical diets, thus being the most susceptible to affect efficiency of protein utilization and ultimately growth (Ball *et al.* 2007). Due to lysine’s role as reference EAA, its requirement should be defined as precisely as possible to avoid biases in the estimation of the other EAA requirements based on the ideal protein approach.

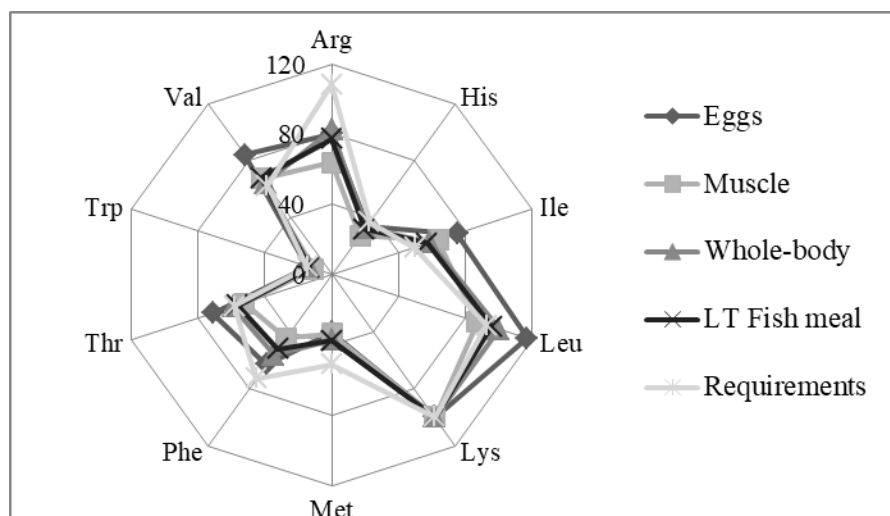


Fig 5. Comparison of EAA profile, expressed relatively to lysine (=100), of fish eggs, muscle and whole-body of different species, LT fish meal and requirement of gilthead seabream (data from Mambrini and Kaushik, 1995; Peres & Oliva-Teles, 2009).

Different EAA profiles have been used as indicative of the optimum dietary EAA profile, such as whole fish egg, whole fish larvae, fish muscle or whole fish carcass (Mambrini and Kaushik, 1995; Fig. 3). Among them, whole-body tissue composition seems to best fit the dietary EAA requirements profile and therefore it is generally used for establishing the optimum EAA profile. This, although whole-body AA reflecting better the EAA required for protein accretion and not taking into account the maintenance AA requirements (Peres and Oliva-Teles, 2009). Nevertheless, maintenance AA requirements usually represents a small fraction of total EAA requirements (Fournier *et al.* 2001; Lall 2005).

The whole-body EAA composition of selected marine fish species is presented in table 7. Mambrini and Kaushik (1995), using a factorial analysis, compared the EAA data available in the literature. These authors observed that although proteins from different tissues have different EAA profiles, the EAA profile of a given tissue seems to converge among species and was not affected by factors such as temperature, feeding rate or fish size. The authors also concluded that the whole-body EAA profile best reflected the optimum EAA profile of a reference protein. Further studies with marine species also suggest that the whole-fish EAA pattern best correlates

with EAA requirements, although it is not completely satisfactory for some fish (Rollin *et al.* 2003; Alam *et al.*, 2005; Luo *et al.* 2008; Peres & Oliva-Teles, 2009). For seabass and Japanese flounder, it was observed that the EAA profile of fish meal reflected better the EAA requirement pattern than the whole-body EAA profile (Alam *et al.* 2002; Peres & Oliva-Teles, 2007; Figure 5). However, for gilthead seabream, Peres and Oliva-Teles (2009) observed that the EAA profile determined by the deletion method correlates tightly to the whole-body EAA composition of seabream.

Table 7. Amino acid composition (g/ 16 g N) of whole-body tissue of some marine fish species¹.

Amino acid	Gilthead Seabream	European Seabass	White seabream	Turbot	Senegalese Sole
Arginine	8.7	8.4	8.6	7.6	6.8
Lysine	7.3	7.6	7.6	6.7	7.3
Histidine	2.8	2.4	2.4	2.3	2.6
Isoleucine	4.1	4.1	3.8	3.6	3.8
Leucine	7.0	7.2	7.2	6.4	7.1
Valine	4.4	4.6	4.2	4.1	4.6
Methionine	2.7	2.6	2.7	2.5	2.7
Phenylalanine	4.4	4.5	4.1	3.2	4.1
Threonine	4.2	4.3	4.1	4.3	4.1
Tyrosine	3.9	3.9	3	3.2	3.1
Aspartic Acid	9.0	9.5	7.9	6.8	10.1
Glutamic Acid	14.1	15.6	13.8	11.8	14.1
Serine	4.5	4.7	4.6	4.8	4.6
Glycine	7.4	7.1	9	9.3	8.2
Alanine	6.3	6.4	6.5	6.4	6.5
Proline	5.1	4.9	4.9	4.9	5.3
Reference	Kaushik, 1998	Kaushik, 1998	Unpublished data	Peres & Oliva- Teles, 2009	Silva, 2009

The optimum EAA profile may also be determined by the deletion method. This method was initially outlined by Wang and Fuller (1989) and is based on the concept that each EAA is

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equally limiting to protein accretion, i.e. body protein accretion rate is directly related to single limiting-EAA and the reduction of a non-limiting EAA has no effect on nitrogen gain. Therefore, changes in nitrogen gain due to partial removal of one EAA in a diet otherwise balanced in its EAA profile will give an estimation of the requirement of that single EAA. The application of this method to all EAA will involve the formulation of a control diet, with a balanced EAA profile, and ten test diets identical to the control diet but replacing about 40-50% of a single EAA by a mixture of non-EAA while maintaining all diet isonitrogenous. The proportional reduction on nitrogen retention of each test diet relatively to the control diet is used to quantify the quantity of each EAA from the control diet that can be removed without affecting N retention (Fig. 6). This approach is largely used in pigs (NRC, 1998), but in fish it was only applied to salmonids (Green & Hardy, 2002; Rollin *et al.* 2003), silvery-black porgy (*Sparidentex hasta*), tilapia (Diogenes *et al.* 2016) and to gilthead seabream (Peres & Oliva-Teles, 2009) (Fig. 7).

Comparison of the EAA profiles obtained by the application of the amino acid deletion method denotes only small differences among fish species. The highest difference is on the arginine requirement, which is higher in gilthead seabream than in salmonids. Similarly, higher arginine requirement per g of nitrogen accretion was also estimated for marine fish species (1.04-1.11 g; gilthead seabream, European seabass and turbot) than for rainbow trout (0.86 g) (Fournier *et al.* 2002).

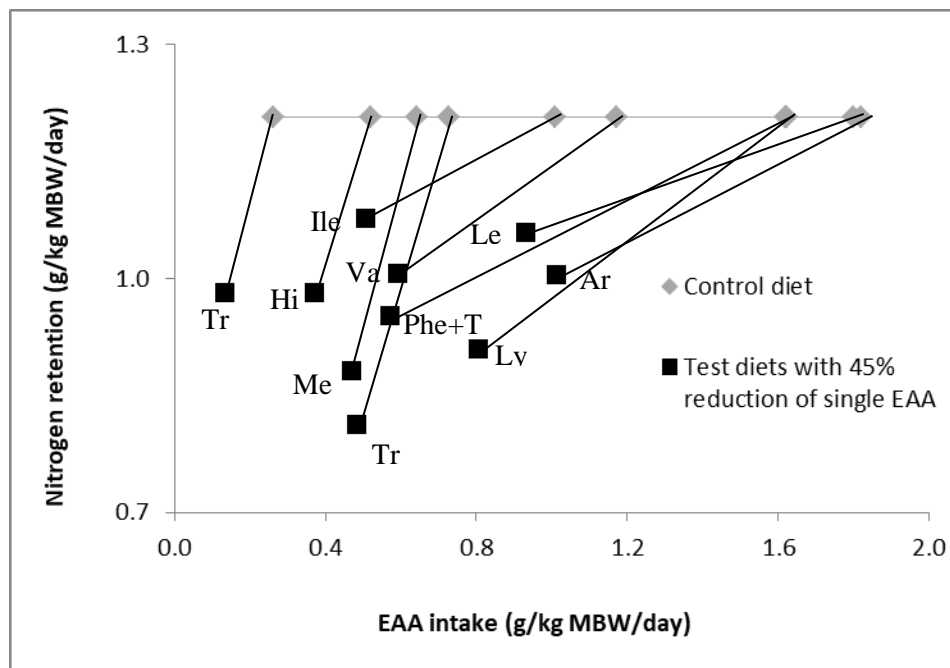


Fig 6. Application of amino acid deletion method: effect of deleting 45% of each EAA from the control diet on nitrogen retention of gilthead seabream (Peres & Oliva-Teles, 2009).

The EAA requirement estimated for different marine fish species using different approaches is summarized in table 8. As it can be seen, for gilthead seabream the EAA requirements estimated by the deletion method or by the whole-body composition are very consistent, except for Phe+Tyr. In fast growing animals.

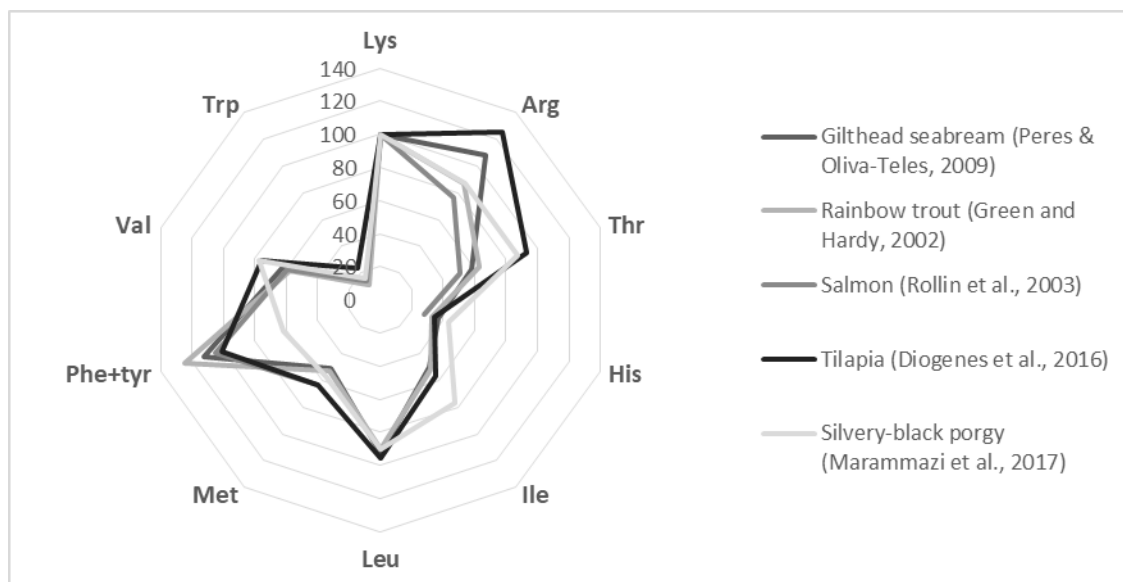


Fig 7. Comparison of the optimum EAA profile, determined by the deletion method, for gilthead seabream, rainbow trout and salmon (data presented relatively to lysine level =100).

Daily accretion of a specific EAA in the whole-body of fed fish has also been used to experimentally determine the EAA requirements. This approach was applied to tilapia, carp, white and Siberian sturgeon (Ogino, 1980; Jauncey *et al.* 1983; Kaushik *et al.* 1991; Ng & Hung, 1995). Although, this method usually provides a good estimate of the optimum dietary EAA profile, its application to estimate AA requirement for growth is questionable. EAA requirement values obtained by this method are usually lower than values obtained by dose-response studies. This is because this method ignores the maintenance requirements and the net efficiency of nitrogen retention that represents only 30 to 40% of the dietary nitrogen.

Given the scarcity of published information on EAA requirement for marine fish species, the application of the ideal protein approach seems a good strategy as it is a fast and practical way to estimate the dietary EAA pattern and to optimize dietary protein level. This information will be highly beneficial for the development of innovative low-cost and low-environmental impact diets by minimizing excess dietary protein level and nitrogen excretion. This information is also critical for a correct formulation of diets based on alternative protein sources to fish meal.

Table 8. Estimation of essential amino acid requirement for selected marine fish species.

Species	Gilthead seabream (<i>Sparus aurata</i>)	Gilthead seabream (<i>Sparus aurata</i>)	Red seabream (<i>Pagrus major</i>)	Common dentex (<i>Dentex dentex</i>)	European seabass (<i>Dicentrarchus labrax</i>)	Turbot (<i>Scophthalmus maximus</i>)	Striped bass (<i>Morone saxatilis</i>)
Method	Whole-body composition	EAA deletion	Whole-body composition	EAA increment	Whole-body composition	Whole-body composition	Whole-body composition
Arg	5.4	5.6	3.5	3.7	4.6	4.8	3.9
His	1.7	1.9	1.4	1.3	1.6	1.5	1.7
Ile	2.6	2.6	2.2	2.5	2.6	2.6	2.5
Leu	4.5	4.8	4.2	4.1	4.3	4.6	5.3
Lys	5.0	5.0	4.4	4.7	4.8	5.0	6.1
Met+Cys	2.4	2.6	2.2	2.2	2.3	2.7	2.8
Phe+Tyr	2.9	5.8	4.1	4.1	2.	5.3	4.7
Thr	2.8	3.0	1.8	2.4	2.7	2.9	3.1
Trp	0.6	0.8	0.6	0.5	0.6	0.6	0.8
Val	3.0	3.2	2.5	2.8	2.9	2.9	2.8
Reference	Kaushik, 1998	Peres & Oliva-Teles, 2009	Forster & Ogata, 1998	Kaushik & Tibaldi, 1998	Kaushik, 1998	Kaushik, 1998	Small & Soares, 1998

Amino acids as functional ingredients

The major fate of AA is towards protein synthesis, a minor proportion being used for different physiological functions but emerging evidence shows that some AA have growth and health promoting proprieties beyond their basic function on body (Wu, 2010a). These AA are known as functional AA, regulating key metabolic pathways that are crucial for maintenance, growth, reproduction and immune responses (Li *et al.* 2007; Wu, 2010a; Andersen *et al.* 2016). Dietary modulation of health, stress, and immune response by AA has already been confirmed in humans and terrestrial animals through dietary supplementation with functional AA at levels above the requirement for growth (Li *et al.* 2007). This approach is particularly interesting as the use of antibiotics and chemotherapeutic treatments in aquaculture is now highly regulated and restricted. Although little is known for fish, recent studies showed that arginine, glutamine, taurine, methionine, tryptophan, proline, and hydroxyproline may have functional proprieties in promoting growth, development and health in fish (Li *et al.* 2007; Andersen *et al.* 2016).

Arginine and glutamine are the best prototypes of functional AA, being able to modulate immune function, cellular redox state and gastrointestinal integrity and, consequently, health status (Li *et al.* 2009; Oehme *et al.* 2010, Andersen *et al.* 2016). Arginine has important regulatory functions in nutrient metabolism and immune response, being of physiological relevance in diseased-animals and in stress situations in which there is a depletion of arginine level in the blood. Moreover, plasma free arginine levels decrease in fish under stressful conditions compared to non-stressed fish (Aragão *et al.* 2008, Costas *et al.* 2008). In fresh water fish, arginine has been reported to be a key-factor of important processes as the production of the pivotal precursor of polyamine synthesis, which enhances fish growth (Li *et al.* 2009; Cheng *et al.* 2012; Andersen *et al.* 2013; Pohlenz *et al.* 2014; Yue *et al.* 2015), the production of nitric oxide which increases immune response and diseases resistance during stress (Buentello & Gatlin, 1999; 2001; Buentello *et al.*

2007; Pohlenz *et al.* 2012a; 2014; Yue *et al.* 2015), and cellular signaling (Bronte and Zanovello, 2005; Holen *et al.*, 2014). Also in fresh water fish species, arginine is known as a more potent insulinotropic metabolite than glucose, increasing the titers of insulin, IGF-1, growth hormone, glucagon and glucagon-like peptide-1 (Mommensen *et al.* 2001; Pohlenz, *et al.* 2013; Andersen *et al.* 2014). In marine fish species, dietary arginine supplementation beyond requirements for protein synthesis was shown to promote growth (Cheng *et al.* 2011) and eliciting positive changes to several components of the innate immune system (Tafalla & Novoa, 2000; Cheng *et al.* 2011; Costas *et al.* 2011; Han *et al.* 2013), oxidative status and intestinal maturation in seabass (Peres *et al.* 1997) and reduced cortisol levels in stress fish (Costas *et al.* 2013). Contrarily, recently it was observed that in gilthead seabream, arginine supplementation has no effect of growth and feed utilization (Coutinho *et al.* 2016b; Oliva-Teles *et al.* 2017), intestinal nutrient absorption capacity, amino acid metabolism and oxidative stress (Coutinho *et al.* 2016b). Also, for golden pompano, it was observed that a surplus of arginine, beyond the requirement level, lead to a reduction of growth performance and protein utilization efficiency (Lin *et al.* 2015). Excessive arginine levels may generate antagonism with lysine, impairing the lysine absorption (Berge *et al.* 1999; Kaushik *et al.* 1988), or may increase ureagenesis (Kaushik & Fauconneau, 1984; Oliva-Teles *et al.* 2017).

Glutamine is considered a pivotal amino acid, regulating several metabolic pathways, including stress and immune responses (Li *et al.* 2009, Andersen *et al.* 2016; 46, 29, 65, 63). Glutamine is utilized as a source of energy for nucleotide synthesis by all rapidly proliferating cells, including enterocytes and lymphocytes. Further, glutamine is known to stimulate muscle protein synthesis in mammals, improve gastrointestinal tract maturation and health. Being the precursor of glutathione, glutamine is also important to protect cells from oxidative stress (Li *et al.* 2007). In human and domestic animals, the benefits of dietary supplementation with glutamine are well documented and include benefits on growth, feed conversion, immune function, cellular antioxidant status and maintenance of

gastrointestinal functions and integrity (Wu *et al.* 2010b). Although little work in this area has been conducted so far in fish, there are recent evidences that open the possibility to extend these benefits to fish production. Studies with tilapia, Jian carp and hybrid striped bass confirmed that diet supplementation with glutamine significantly increased growth, intestinal function and structure (Yan & Qiu-Zhou, 2006; Lin & Xiau, 2006; Silva *et al.* 2010; Cheng *et al.* 2012; Jiang *et al.* 2015; Hu *et al.* 2015). Moreover, it has been observed that glutamine supplementation increased macrophage production of superoxide anion, neutrophil oxidative radical and lysozyme activity (Cheng *et al.* 2011; 2012) and has a protective effect against the oxidative stress in intestinal epithelial cells and hypoxia stress (Chen *et al.* 2009; Liu *et al.* 2015). It was also confirmed that dietary supplementation with arginine plus glutamine significantly increased feeding rate and growth during the first autumn (Oehme, *et al.* 2010). *In vitro* studies also showed that glutamine promoted fish enterocytes growth (Jiang *et al.* 2009) and increased non-specific T and B cell proliferation (Pohlenz *et al.* 2012b). Recently, it was observed that growth and feed utilization of gilthead sea bream juveniles was not affected by the dietary glutamine supplementation (Caballero-Solares *et al.* 2015; Coutinho *et al.* 2016), but it increased protein retention (Caballero-Solares *et al.* 2015), and modulated hepatic and intestinal antioxidant responses (Coutinho *et al.* 2016c).

Beyond their roles as substrates for protein synthesis, methionine and cysteine are involved in numerous roles in metabolism, being methyl donor for several methylation reactions, including DNA, and precursors of important molecules, such as glutathione and taurine, which are essential compounds in defense mechanisms against oxidative stress (Métayer *et al.* 2008; Andersen *et al.* 2016; Coutinho *et al.* 2017). In juvenile hybrid striped bass, it was observed that methionine deficiency in diet might exhaust reservoirs of non-enzymatic antioxidant defenses such as ascorbic acid, vitamin E or glutathione, and increase liver lipid oxidation. Oxidation of methionine residues also acts as scavenger of reactive oxygen species (Weissbach *et al.* 2005; Métayer *et al.* 2008; Feng *et al.* 2011).

Together, these negative repercussions may result in oxidative stress (Li *et al.* 2009; Andersen *et al.* 2016). Under stress situations it was reported a decrease of plasma free methionine, suggesting an increased use of this AA, probably related to the increased demand for glutathione and taurine (Aragão *et al.* 2008; Costas *et al.* 2010). However, in gilthead bream dietary methionine supplementation above the requirement level was not required to maintain normal hepatic glutathione levels (Perez-Jimenez *et al.* 2012). In European seabass it was showed to modulate the oxidative stress response but did not affect growth performance (Coutinho *et al.* 2017).

Taurine and hydroxyproline are non-protein AA that are abundant in fishmeal but not in plant protein sources. Taurine is considered a conditionally EAA, specially for marine fish species fed low-fishmeal based diets, and is involved in important biological functions, such as fat digestion, bile salt conjugation, antioxidant defense, osmoregulation, as well as development of visual, neural and muscular systems (Li *et al.* 2009; El-Sayed, 2014; Salze *et al.* 2015). Capacity to biosynthesize taurine depends on fish species, and for some carnivorous species supplementation of low-fish meal diets with taurine is required to maximize growth (Goto *et al.* 2001, 2003; Park *et al.* 2002; Matsunari *et al.* 2005, 2008; Takagi *et al.* 2010; Jirsa *et al.* 2014; Wu *et al.* 2015; Lopez *et al.* 2015; Al-Feky *et al.* 2016; Li *et al.* 2016b). Taurine-deficient diets are also known to impaired liver function (El-Sayed, 2014; Salze *et al.*, 2015), inducing green liver symptom is carnivorous fish species such as red seabream, yellowtail and Japanese flounder (Takagi *et al.* 2006, 2008, 2011; Goto *et al.* 2001). The role of taurine on lipid digestion and formation of bile salts is well documented (Salze & Davis, 2015). In fish, bile acids conjugations occur mainly with taurine; conjugated bile acid are actively absorbed distal intestine, thus maintaining adequate concentrations in most of the intestine (Salze & Davis, 2015). Low taurine level may impair conjugation of bile acids and so lipid digestion. Evidence shows that taurine supplementation increases bile salt content (Kim *et al.* 2007, 2015; Nguyen, *et al.* 2015),

although it appears to be independent from dietary lipid levels (Kim *et al.* 2008; Salze *et al.* 2015).

Increased evidences have also highlight the role of taurine as an antioxidant (El-Sayed, 2014; Salze *et al.* 2015). Taurine depletion may induce oxidative and inflammatory stress (El-Sayed, 2014; Salze *et al.* 2015). Taurine antioxidative action may be associated to its capacity to act as a non-specific scavenger of reactive oxygen species, to modulate the production of reactive oxygen species or to improve the antioxidant enzyme activity (Divakaran, 2006; Aydogdu *et al.*, 2007; Banuelos-Vargas *et al.* 2014; Feidantsis *et al.* 2014; Han *et al.* 2014). Dietary taurine supplementation reduced lipid peroxidation (Zhang *et al.* 2004; Aydogdu *et al.* 2007; Chang *et al.* 2011; Sevgiler *et al.* 2012; Hammes *et al.* 2012; Banuelos-Vargas *et al.* 2014). Taurine has also been reported to have some health benefits, including alleviation of metal toxicity (Gurer *et al.* 2001; Sevgiler *et al.* 2011; Hammes *et al.*, 2012) and resistance against hyperammonemia (Li *et al.* 2016).

Tryptophan is the only amino acid that can be converted into serotonin, the precursor of melatonin, having several important biological functions such as appetite regulation, immune response and health maintenance (Andersen *et al.* 2016). Some studies with animals, including fish, indicate that tryptophan is a rate limiting factor for serotonin synthesis, inducing a dose-dependent response (Johnston *et al.*, 1990; Herrero *et al.*, 2007). Consequently, and although the rate limiting step for melatonin synthesis is modulated by the enzymes AANAT and HIOMT, these reactions are dependent on substrate concentration and therefore, tryptophan also acts as limiting factor for melatonin production (Huether *et al.* 1992). Supplementing dietary tryptophan reduced aggressive behavior in different fish species (Winberg *et al.* 2001; Hodlund *et al.* 2005; Clotfelter *et al.* 2007). Tryptophan, through its precursors, serotonin and melatonin, is known to act as a free radical scavenger, playing key roles on cell redox balance by promoting the activity of antioxidative enzymes, which was also observed in fish species (Wen *et al.* 2014; Ciji *et al.* 2015). Recently for Senegalese sole it has shown that tryptophan has immunomodulatory

properties after a bacterial challenge (Azeredo *et al.* 2016), while the opposite was observed for seabass (Machado *et al.* 2015). In carp, it was observed that an appropriate dietary tryptophan level improves grass carp growth, intestinal immune response, barrier function and antioxidant status, and regulated the mRNA levels of related signal molecules, whereas as higher levels of tryptophan severely reduced growth (Wen *et al.* 2014). Dietary fortification with tryptophan also modulates growth and immuno-metabolic status of *Labeo rohita* juveniles exposed to nitrite (Ciji *et al.* 2015).

Proline and hydroxyproline are also considered conditionally EAA for some fish in both early life and adult stages, also having functional properties related to antioxidant and immune responses (Wu *et al.* 2011). Hydroxyproline is produced by post-hydroxylation of proline after protein synthesis. Dietary supplementation of low-fishmeal diets with hydroxyproline improved growth and collagen formation (Aksnes *et al.* 2008). However, for turbot, hydroxyproline supplementation of plant-based diets had no effect on growth, but increased tissues Hyp and muscle total collagen concentration (Zhang *et al.* 2013).

Conclusions

Diet formulation based on an adequate protein content and optimum EAA profile is a key factor to improve efficiency of AA utilization for protein accretion, therefore reducing nitrogen excretion. However, information on protein requirement and particularly on the requirements of the 10 classically considered EAA is limited for the majority of marine fish species with interest for aquaculture. This lack of information is of practical relevance when considering replacement of fishmeal by plant protein as main dietary protein sources, due to the potential unbalance of EAA that may occur.

Up to now, studies on EAA requirements of marine fish have been focused on a limited number of EAA, namely lysine, methionine and arginine, which are EAA that may be the first limiting in the most commonly, used feed ingredients. Recently, and as consequence of the use of plant feedstuffs as main dietary protein sources, particular attention has been given to taurine requirements. Estimation of EAA requirement based on the ideal protein concept is a highly versatile approach, allowing the estimation of the requirement of all EAA based on the determination of the requirement of just one EAA and the ideal protein EAA profile. Due to the high diversification of new marine species with interest for aquaculture, the ideal protein approach may be used as the first approach to provide fast and relatively inexpensive information of the EAA requirements.

The major proportion of AA is used for protein synthesis; a minor proportion being used for different metabolic purposes. Related to this, emerging evidence shows that some AA when incorporated at the diets at a level above requirements may have functional properties, regulating key metabolic pathways crucial for maintenance, growth, reproduction, oxidative status and immune responses of the animals. Further studies are needed to elucidate the AA induced changes and their potential application as modulators of growth and well-being of fish.

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Role of Marine Metabolites in Shrimp Growth, Production and Disease Prevention

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Abstract

This review consolidates the recent information about the global aquaculture production status of fish and shellfish. Research reports on common and emerging microbial diseases of cultivable species of shrimp are narrated together with resultant reductions in productivity. The harmful effects antibiotics usage in shrimp aquaculture and consequent effects on the species as well as ecosystem are highlighted. The *in vitro* and *in vivo* tests of extracts containing metabolites isolated from marine macroalgae, sea grasses and invertebrates in restraining the proliferation of disease causing microbes are presented. The metabolite bearing marine species and methods of extraction of marine metabolites are concisely indicated. Studies on unique bacteria associated with the metabolite - bearing marine organisms, their identity and their significance in metabolite production are essential for future research and developmental activities in aquaculture and pharmaceutical applications. The administration of marine metabolites in the post larval and juvenile shrimps as feed additive for controlling the common microbial diseases such as vibriosis together with essential administration protocols are reported. Many of the tested marine metabolites are of immense use as they increased the survival of farm reared shrimps and their growth rates. The resultant specific growth rate attained together with survival contributed to the significant increase in production. Perusal of literature indicated that marine secondary metabolites isolated from marine macro algae and sponges exhibited immune enhancing activity in shrimps in a non specific manner when administered as a feed ingredient.

Key words: Disease management, growth promoter, immune enhancer, marine metabolites, shrimp aquaculture

Introduction

As per the recent estimates of FAO (2016), the production of aquatic animals from aquaculture in 2014 amounted to 73.8 million tonnes, with an estimated first-sale value of US\$160.2 billion. This total comprised 49.8 million tonnes of finfish; 16.1 million tonnes of molluscs; 6.9 million tonnes of crustaceans and 7.3 million tonnes of other aquatic animals including amphibians (US\$3.7 billion). China accounted for 45.5 million tonnes in 2014, or more than 60 percent of global fish production from aquaculture. Other major producers included: India, Viet Nam, Bangladesh and Egypt. These statistics indicated that presently, the global aquaculture production has been increasing consistently. Feed is widely regarded as a major constraint factor to the growth of aquaculture production in many developing countries (FAO, 2016). The other constraint or problem for successful aquaculture is the disease outbreaks and consequent losses. In shrimp farming, intensive culture conditions increase the risks of waste accumulations, consequent stress for the stocks and proliferation of pathogens. The outbreak of viral diseases has increased the economic risks and slowed the shrimp farming entrepreneurship and industry development (Flegel, 2006). According to Stentiford *et al.* (2012) Disease in aquaculture will certainly limit future food supply from global crustacean fishery and aquaculture sectors. In a recent publication by Stentiford *et al.* (2017) new paradigms to help solve the global aquaculture disease crisis were stated with special reference to shrimp farming in Thailand.

It is well known that aquatic environments impose a constant and omnipresent risk of pathogen exposure to resident hosts, perhaps even more so than terrestrial systems (Oidtmann *et al.* 2013). Poor knowledge of background microbial diversity in farm systems leads to frequent emergence of previously unknown pathogens, surprising farmers and creating shock in the wider value chain (Lightner *et al.* 2012; Flegel, 2012 and Shinn *et al.* 2014). High throughput sequencing (HTS) applied to open aquatic systems is rapidly increasing our knowledge of prokaryotic and eukaryotic diversity and the complex symbiotic arena in which they exist (Karsenti *et al.* 2011). According to Bass *et al.* (2015), application 'environmental DNA' (eDNA) approaches to aquaculture pond systems with reference to the disease outbreak and non-outbreak systems will provide important

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information about by detecting specific pathogens of consequence to farmed hosts or those elements of the microbiome that facilitate their emergence as disease agents. Thus, the improved definition of a ‘pathobiome’ within hosts may be expected to supersede an historic focus on specific pathogens as sole perpetrators of yield-limiting disease (Gilbert, 2016). A shift from single-pathogen to pathobiome concepts may also expose a wider target to which pond management strategies can be applied (De Schryver, 2014). It becomes imperative to apply modern HTS approaches in order to accelerate the understanding of the complex trophic structures that exists within such systems and the health outcomes for farmed species stocked (De Schryver, 2014).

In shrimp aquaculture, infectious diseases are causing particularly devastating economic and social impacts, with total losses exceeding 40% of global capacity (Israngkura and Sae-Hae, 2002). Emergent diseases, often with cryptic or syndromic aetiology (such as early mortality syndrome in shrimp), have collapsed production in nations across Asia (Lee C-T *et al.* 2015), confirming disease as the major constricting factor for expansion of the aquaculture industry to 2050 (Stentiford *et al.* 2012) Increasingly globalised trading of seafood between net exporting and importing nations expands the geographical range over which these effects are felt (Jennings *et al.* 2016). In this context, 50 early-career scientists from the United Kingdom and Thailand met with industry professionals and policymakers in March 2016 to consider the future challenge of managing disease in global aquaculture and to discuss new paradigms for mitigating their negative effects. This Opinion summarises major outcomes of those discussions and proposes a need to refocus strategic scientific and policy priorities relating to aquatic animal health in support of an expanding and sustainable industry to 2050.

Impacts of antibiotics

Application of antibiotics and other chemicals in shrimp aquaculture has its own intricate problems. For example, regular use of antibiotics in shrimp hatchery or grow out system may lead to development of not only antibiotic resistant fish/shrimp bacteria, but also human bacteria. The presence of antimicrobial residues in products of aquaculture is a

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threat to human health. Information is still lacking on the absorption and distribution of antibiotics in fish and shrimp and persistence of residues or effects of them in the environment. Hence, promoting the holistic systems approach in managing shrimp health problems needs special attention (Selvin *et al.* 2009).

Considering the potential threat of diseases on the one hand and the environmental issues on the other hand, disease management aspects should concentrate on environment friendly strategies. Proactive disease managing techniques are to be evolved as they are essential to protect the aquacultured shrimps from emerging and virulent strains of microbes. Researchers have reviewed the potential benefits of probiotics, which could be a promising alternative for antibiotics in aquaculture, demonstrating beneficial effects to host by combating diseases, improving growth and also stimulating immune responses of host toward infections (Newaj-Fyzul *et al.* 2014; Hai, 2015).

Common shrimp diseases

Shrimp aquaculture mostly concentrated on species such as *Penaeus monodon* and *P. vannamei*. Both these species are known to be vulnerable to a wide range of microbial diseases including viral, bacterial, fungal and protozoan diseases. Among viruses, IHHNV, YHV, TSV, WSSV, and IMNV are considered as major ones affecting shrimps. Their distribution pattern, pathology, morphology, and genomic organization, diagnostic methods and common intervention practices were reported by Seibert and Pinto (2012). Mass mortalities and failure of the culture system have also been recorded. Of late, *P. vannamei* has been cultivated throughout the world. The common disease of economic importance reported to affect *P. vannamei* include: WSD, infectious hypodermal and hematopoietic necrosis (IHHN), Taura syndrome (TS), yellow head disease (YHD) and infectious myo necrosis (IMN).

WSD caused US\$6billion loss. IHHNV has caused about US\$0.5-1 Billion loss in America. The impact of TS on the shrimp-farming industry in the America was estimated to be US\$ 1-2 billion up to 2001 while in Asia it is of \$0.5-1 billion. An unpublished data

form Brazilian shrimp farmers' association has estimated that the loss due to infectious myo necrosis IMN from 2002 to 2006 in Brazil exceeded \$100 million. The new unique disease, Acute Hepato Pancreatic Necrosis disease or AHPND earlier known as early mortality syndrome (EMS) has been devastating *P. vannamei* farms in China since 2009, Vietnam since 2010, Malaysia since 2010, and Thailand since 2012, where in 100% mortalities have been reported during the first 20-30 days after stocking. The AHPND has caused about 60% drop in shrimp production in the affected region compared with 2012 and the global estimate of the loss per year is about US\$1 billion.

In Indonesia this IMNV caused significant losses exceeding \$1 billion by 2010. A total of \$100-200 million loss has occurred mainly because of the disease in America, but in Asia this disease has emerged in the year 2006, and the estimated loss is about \$1 billion. Significant loss from YHD amounts to \$1 billion per year in Asia. Since 2012, Thai shrimp farmers have suffered major economic losses owing to Early Mortality Syndrome (EMS). The disease reduced shrimp production of Thailand from 5,40,000 tons in 2012 to 2,56,000 tons in 2013 and 2,10,000 tons in 2014, respectively (Chuchird *et al.* 2015). The pathogenic *Vibrio parahaemolyticus* was suspected to be associated causing mass mortality as it induced 100 % mortality with typical EMS pathology to experimental shrimp (Tran *et al.* 2013).

In Mexico, Escobedo-Bonilla (2016) had narrated about the emerging microbial diseases. Several bacterial viral agents were recorded to reduce production in shrimp farms. Bacterial diseases include vibriosis caused by different *Vibrio* species, a rickettsia-like bacteria cause necrotizing hepatopancreatitis (NHP-B), filamentous bacteria contribute to surface and gill fouling of shrimp as well as chitin-degrading bacteria provoke cuticle necrosis. Bacterial pathogens causing this injury include *Vibrio* sp., *Aeromonas* sp., *Spirillum* sp. and *Flavobacterium* sp. In hatcheries, a bacterial disease probably caused by *Vibrio harveyi* induced high mortalities to larval shrimp in zoea II stage (Escobedo-Bonilla, 2016). In Mexico, major viral pathogens which caused high mortalities in shrimp included Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV or Penstyldenovirus), Taura Syndrome Virus (TSV), Yellow-Head Virus (YHV) and White Spot Syndrome Virus (WSSV) (Escobedo-Bonilla, 2016).

In Latin America, presumptive systemic streptococcal infections were detected histologically in farmed *P. vannamei* juveniles. Differing *Streptococcus* identifications were obtained using API 20 Strep and Biolog systems, the former identifying the isolate as *Streptococcus uberis* and the latter as *S. parauberis*. Injection of specific pathogen-free (SPF) *P. vannamei* with the bacteria resulted in 100% mortality by 3 d post-injection with successful recovery of the agent from moribund test shrimp hemolymph samples. The recovered isolate was used in per os and waterborne exposure studies of SPF *P. vannamei* with mortalities ranging from 40 to 100% and 80 to 100%, respectively. These findings were described as the first reported case of streptococcosis in marine penaeid shrimp in the West by Hasson *et al.* (2009).

From India, Selvin *et al.* (2009) gave a comprehensive account about the shrimp diseases and their management options. Most of their studies were on *Penaeus monodon*. In the hatcheries, occurrence of white tail disease (WTD) caused by MrNV and XSV in hatchery-reared post-larvae of *Penaeus indicus* and *P. monodon* were reported by Ravi *et al.* (2009). Recently, Gunalan *et al.* (2014) reviewed the disease occurrence among *Litopenaeus vannamei* in the shrimp culture systems from different geographical regions of India. Their observation indicated minor regional differences in the six major disease outbreaks in the *L. vannamei* culture systems. Most of the Black gill disease occurrences were in Andhra Pradesh; IHHNV in Andhra Pradesh and Orissa (north eastern coastal areas); WMD in Tamil Nadu and Andhra Pradesh; White gut disease in Tamil Nadu and Andhra Pradesh; Muscle cramp disease in Tamil Nadu and Andhra Pradesh.

Marine Metabolites in Shrimp Disease Control Strategies:

Marine Macro algae:

Lipton and Jean Jose (2006) initially reported about the immune enhancing activity of marine macro algae and their applications in shrimp farming as a non specific immune enhancer. Lipton *et al.* (2009) evaluated the macroalgal feeding strategies in improving the expulsion of pathogens from the hemolymph of shrimps administered with medicated feed and subsequent experimental infection by pathogenic vibrios. Their studies clearly

indicated that the incorporation of macroalgal extract in feed to shrimp has successfully cleared the pathogenic bacteria faster from the hemolymph of *Penaeus monodon*. Impact of marine secondary metabolites (MSM's) from *Hypnea musciformis* as an immunostimulant on haemogram count and *Vibrio alginolyticus* infection in *Penaeus monodon* at different salinities were elaborated by Jose, Lipton and Subhash (2008). Shrimp pathogenic vibrios like *Vibrio alginolyticus* and *V. fischeri* were inhibited by the methanolic extract of *H. musciformis*. Simultaneous studies by Huxley and Lipton (2009) inferred that the marine macroalgae *Sargassum wightii* also provided immunomodulatory effect in *P. monodon*. Hemolymph parameters such as phagocytosis, agglutinin and bactericidins in the hemolymph were evaluated to substantiate the effect.

Shrimp disease management using macro alga *Ulva* diet was proven to be an effective eco-friendly management strategy for sustainable shrimp farming (Selvin *et al.* 2011). The *Ulva* diet was found to be a potent immunomodulator and therefore it was considered as a proactive drug. As per their earlier finding, 88% of viable *V. fischeri* cells were cleared-off from the haemolymph within 1 h in the *Ulva* treated group. These findings suggest the quick production of bactericidins in the haemolymph of *Ulva* treated group. Thus, the rapid bacterial clearance rate of shrimp haemocytes was stimulated by feeding with *Ulva*. Therefore, it was conjectured that bacteridins found in shrimp plasma might be induced released from haemocytes by *Ulva* diet. Huang *et al.* (2006) reported the effect of seaweed *Sargassum fusiforme* polysaccharide extracts on vibriosis resistance and immune activity of the treated shrimp. Literature also evidenced the *in vivo* antiviral (WSSV) potency of seaweed-based medicated feed in *Penaeus monodon* (Manilal *et al.* 2009). Selvin *et al.* (2011) mentioned about the easy preparation of the *ulva* diet for shrimps from the raw extract of the macroalgae. They also reiterated that purification strategies and consequent synthetic analogue development process need not be undertaken by any shrimp farmer for preparing the diets. As the extract was prepared from the dried material, such source material can easily be stored for 12 months. To sustain the host-defense system and relative protection against pathogenic invaders, the *Ulva* medication can be used as a prophylactic agent for the entire culture period at minimal expenditures. Based on the findings, it was concluded by them that the secondary metabolites of *U. fasciata* form an

excellent source for developing potent formulations as a package of proactive management practice for sustainable shrimp farming.

As the ulva medicated feed was proved to be one of the major non specific immune modulator and inhibiting vibriosis in shrimps, several studies followed in marine macro algal extracts. Chakraborty, Lipton and team (in 2011) fractionated the raw organic extracts of *U. fasciata* extracts using standard methods. Interestingly, their reports revealed that the Guaiane sesquiterpenes isolated and purified from the *Ulva fasciata* extract is a major compound exerting antibacterial properties.

Further studies by a team of researchers evaluated the antimicrobial potential of marine organisms collected from southwest coast of India against multi-resistant human and shrimp pathogens (Manilal *et al.* 2010). (Wulfen) Lamouroux and *Hypnea valentiae* (Turner) Montagne with specific activity Pramitha and Lipton (2013) evaluated the antibiotic potentials of red macroalgae *Hypnea musciformis* against fish and shrimp pathogenic bacteria. It is interesting to note that the aqueous extract of many of the common tropical macroalgae had antibiotic activities (Chritobel & Lipton, 2011). In vivo studies on the therapeutic values of marine macroalgae against the fish pathogen *Aeromonas hydrophila* (Celia & Lipton, 2012).

Cells responsible for removing foreign material include circulating hemocytes and fixed phagocytes, primarily in the gills and digestive gland (Lipton *et al.* 2011). Humoral factors such as non-self recognition proteins, prophenoloxidase, and antimicrobial peptides are produced, stored, and released from hemocytes. Hemocytes can adhere to a pathogen, triggering phagocytosis and thereby producing highly toxic reactive oxygen species which help eliminate foreign particles. The humoral and cellular defense mechanisms of shrimp can be assessed by bacterial clearance assays. The bacterial clearance capability of shrimp was steady in the control and experimental groups from day 40 onwards, indicating an age-dependent pattern and that shrimp hemolymph attains a high capability for removing invading pathogens. The bacteria were ultimately cleared in all treatments including the control, but the rate of clearance was improved by the medicated feed. These data support

the idea that rapid clearance of live bacteria, whether by bactericidal mechanisms in the hemolymph or by physical trapping and removal to peripheral sites, contributes to disease resistance in shrimps by limiting the spread of free pathogens to other tissues. The use of marine secondary metabolites from *H. musciformis* during the juvenile stage of *P. monodon* can assist in such clearance (Lipton *et al.* 2011).

Dashtiannasb *et al.* (2016) and Dashtiannasb & Yeganeh (2017) evaluated the effect of ethanol extract of a macroalgae *Laurencia snyderia* on growth parameters and vibriosis resistance in shrimp *Litopenaeus vannamei* in Iran. Their growth and survival data indicated that the mean survival was maximum ($95.5 \pm 3.4\%$) for those shrimps that received enriched Artemia at 0.6 mg mL^{-1} concentration. The survival shrimps fed enriched Artemia at 0.2 and 0.4 mg mL^{-1} concentration were $92.22 \pm 3.2\%$ and $92.22 \pm 5.2\%$ respectively. In the control set, a minimum survival of 91.33% was recorded. The weight gain of $333.2 \pm 3.3 \text{ mg}$ was noted in the experimental group fed with enriched artemia with 400 mg mL^{-1} . But the minimum weight gain of $237.4 \pm 4.6 \text{ mg}$ was displayed in control group that was significantly different. The specific growth rate (SGR) of the *L. vannamei* juveniles after 30 days culture in different treatments and control varied with a statistically significant increase in the experimental treated group. The group also showed high resistance towards induced vibriosis with *Vibrio harveyi* challenge.

Crude extract prepared from the red seaweed *Laurencia snyderiae* obtained from the Persian Gulf was evaluated for shrimp growth performance and to determine *in vivo* efficacy of this seaweed in the prevention of shrimp vibriosis. The ethanol extract from *L. snyderiae* (EELS) that was fed to the *Artemia* instar I for their enrichment was found to be non toxic to them. Subsequently, juvenile shrimps of *Litopenaeus vannamei* were fed with these enriched *Artemia* at 0 mg mL^{-1} (Control group), 200 mg mL^{-1} , 400 mg mL^{-1} and 600 mg mL^{-1} for 30 days. The results obtained showed a significant increase ($p < 0.05$) in survival rate in treatment groups compared with that in the control group. Shrimps fed with enriched *Artemia* showed a significant improvement in growth parameters when compared to those in the control group. When these juvenile shrimps were exposed to *Vibrio harveyi* (after 30 days) they showed notably lower mortality than the control. These results indicate that EELS has a good potential

in promoting growth and antibacterial activities against *V. harveyi* that is useful in shrimp aquaculture (Dashtiannasb *et al.* 2016 and Dashtiannasb & Yeganeh, 2017).

Sea grasses:

Apart from macroalgae, sea grasses and their associated bacteria were also reported to exhibit antibiotic activities, including *Vibrios* from aquaculture sites (Merina and Lipton, 2010). Incorporating their cell free cultures or exo cellular products in the feed will be of immense use in the aquaculture industry and further studies are required in this direction.

Marine Sponge metabolites and sponge - associated bacterial metabolites:

Secondary metabolites from five sponge species inhibited the growth of eight virulent marine fish pathogens (Annie *et al.* 2008, Annie & Lipton, 2012). Antimicrobial Potential of the Marine Sponge *Sigmatocia pumila* from the South Eastern Region of India by Dhinakaran and Lipton (2012a) provided clues for developing therapeutics against common bacterial pathogens of shrimps and fishes. In addition to sponge extracts, the culturable bacteria from sponges were isolated and evaluated for bioactivity. Several initial studies on the sponges and their associated bacteria towards controlling infectious microbes of fish and shellfish were accomplished by Lipton and his team (Lipton *et al.* 2014 a, b). Bacteria were isolated from different sponges collected off Kanyakumari (Lat: 8°4'60N; Long: 77°34'0 E) and Vizhinjam (Lat: 8°22'45N; Long: 76°59'0 E) coasts of Southern India. A new media incorporating each specific sponge extract was employed to retrieve maximum groups as well as numbers of bacteria from *Callyspongia diffusa*, *C. subarmigera*, *Clathria gorgonoides*, *Echinodictyum gorgonoides*, *Ircinia fasciculata*, *Phloeodictyon sp.*, *Sigmatocia carnosus*, *Spongia officinalis*, *Thalysias procera* and *T. vulpina*, *Zygomycete angulosa*. In the sponge extracted media, a higher bacterial numbers of 4.8×10^6 CFU/ml with 12 distinct colonies were retrieved from *C. subarmigera* compared to 6.4×10^5 CFU/ml with 5 distinct colonies in normal media. *Bacillus rubidae* (GenBank: JN873082) and *Serratia amyloliquefaciens* (GenBank: JN873083), and *Arthrobacter sp* (GenBank: JN 873081) retrieved from *C. subarmigera* tissue exhibited high antibiotic activities than the conventional antibiotic discs. Pathogenic fish and shellfish isolates such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus mirabilis*, *Vibrio*

alginolyticus and *V. harveyi* were inhibited by the cell free culture extract of the sponge-associated *Bacillus* and *Serratia*. In another series of tests, the sponge tissues of *Echinodictyum gorgonoides* yielded 3.2×10^6 CFU/g of bacteria with 5 distinct genera at the pH optimum of 8.0. The cell free culture filtrate of the isolates has maximum activity towards *Bacillus subtilis*, *S. aureus* and *P. aeruginosa*. The culture filtrates of the bacteria exhibited similar activities as that noted from the organic extracts of the whole host sponge tissue extracts (Dhinakaran & Lipton, 2012b).

A recent study of the cell free supernatant of sponge *Callyspongia diffusa* - associated bacteria showed significant antibacterial activity against most of the fish and shellfish pathogenic isolates. Molecular characterization of one of the potent isolate showed a lineage with *Shewanella algae* (Rachanamol *et al.* 2014). Almost all the sponge isolates and cell free supernatants also showed activity against *V. harveyii*, *V. fluvialis*, *V. anguillarum*, *Proteus vulgaris* and *L. lactis*. Such vibriostatic efficacy of *Shewanella algae* isolated from *Penaeus monodon* against *V. parahaemolyticus* and *V. alginolyticus* was reported by Shakibazadel *et al.* (2008) and this was statistically proved.

Of late there are supply problems and the utilization of metabolite producing sponge specific bacteria will be of immense use in drug developments. In addition, Lipton and Shine (2010) developed mariculture techniques to grow the explants tissues of sponges for easy harvesting and extraction of metabolites. Sponges such as *Callyspongia subarmigera* (Ridley) and *Echinodictyum gorgonoides* (Dendy) were cultured and the extracts of cultured sponge exhibited antagonistic effects towards fish and shellfish pathogenic bacteria as noted in the natural habitat collected sponges.

The results of the cell free supernatant of associated bacteria showed that all the bacterial isolates of *Callyspongia diffusa* showed a significant antibacterial activity against most of the fish and shellfish. The isolate The BLAST search of the 16S rRNA gene sequence the isolate showed that the one of the potent isolate showed a lineage with *Shewanella algae* (Rachanamol *et al.* 2014). Almost all the sponge isolates and cell free supernatants also showed activity against *V. harveyii*, *V. fluvialis*, *V. anguillarum*, *Proteus*

vulgaris and *L. lactis*. Such vibriostatic efficacy of *Shewanella algae* isolated from *Penaeus monodon* against *V. parahaemolyticus* and *V. alginolyticus* by Shakibazadel *et al.* (2008) was statistically proved.

Probiotics

Probiotics have been proven to be positive promoters of aquatic animal growth, survival and health. In aquaculture, intestines, gills, the skin mucus of aquatic animals, and habitats or even culture collections and commercial products, can be sources for acquiring appropriate probiotics, which have been identified as bacteria (Gram-positive and Gram-negative) and nonbacteria (bacteriophages, microalgae and yeasts). While a bacterium is a pathogen to one aquatic animal, it can bring benefits to another fish species; a screening process plays a significant role in making a probiotic species specific (Hai, 2015).

Potential probiotics may be commonly obtained from various sources viz. the GI tracts of aquatic animals (Del'duca *et al.* 2013, Beck *et al.* 2015) and fish mucus (Tapia-Paniagua *et al.* 2012). Particularly they are the collected cultures (Thompson *et al.* 2010) and commercial products (Suzer *et al.* 2008). In marine bivalve hatchery, Subhash *et al.* (2007) studied about the role of probiotics in pearl oyster hatcheries. Their studies revealed the influence of probiotic bacterium *Lactobacillus acidophilus* on the enhanced survival and growth of pearl oyster *Pinctada fucata* spats.

Exclusion of *Vibrio* spp. by an antagonistic marine actinomycete *Streptomyces rubrolavendulae* M56 was reported by Augustine *et al.* (2015) from India. Subsequent review by Tan *et al.* (2016) gave an insight in to the use of the genus *Streptomyces* bacteria as an alternative to antibiotics, being a probiotic in controlling diseases and improving the health and quality of aquaculture production. The prospects and limitations of *Streptomyces* species as a probiotic in aquaculture were also discussed by Tan *et al.* (2016).

In white shrimp *Litopenaeus vannamei* and *Fenneropenaeus indicus* vast strains of *Bacillus* have been tested as probiotics in order to improve dry matter digestibility, phosphorus, and crude protein. Consequences of *Bacillus* administration with a dose of 50 g kg⁻¹ feed revealed higher growth sizes (Cook *et al.* 2006). Other research has suggested the importance of managing the probiotic in all ontogenetic stages of the shrimp to generate a constant effect on the production of digestive enzymes (Strozzi, L. Mogna, 2008). In order to develop a potent endogenous probiotic from shrimp, screening of digestive canal bacteria of health *Litopenaeus vannamei* resulted in four species, they were identified as *Bacillus megaterium* BM1, *Bacillus firmus* BM2, *Actinobacillus* spp. BM3 and *Pseudomonas stutzeri* BM4. *B. megaterium* BM1 was the ideal probiotic candidate for enhancing growth on *L. vannamei*, it resulted in production of digestive extra cellular enzymes and a premium value of steady growth rate. Concentration of 10⁶ cells g⁻¹ diet from *B. megaterium* BM1 in an *in vivo* study resulted in beneficial effects for the growth and feed utilization of *L. vannamei* (Yuniarti *et al.* 2013).

Apart from products from bacteria, fungal metabolites were also evaluated for shrimp growth and disease control. Wahjuningrum *et al.* (2016) reported better survival rate, daily growth rate and feed conversion ratio in shrimps treated with *Nodulisporium* sp. KT29. The fungal *Nodulisporium* sp. KT29 metabolites contained bioactive compounds including 0.54% β -(1,3) glucan and phytochemical compounds (121 ppm of phytosterol, 23 ppm of saponin and 31 ppm of polyphenol Saputra *et al.* (2016). The presence of phytochemical substances, β -glucans and polyphenols were inferred for the immunostimulants and antioxidants activities and negative effects of stress, increase diseases resistance, and improve various physiological performances (Shelby *et al.* 2007; Welker *et al.* 2007, Soltanian *et al.* 2009; Kiron 2012). According to Hai & Fotedar (2009), the administration of β -glucan on shrimp caused the structure of intestinal surface becoming wider, so that the nutrients absorption became better. The improvement on the digestion and the nutrients absorption will cause the enhanced feed efficiency and protein absorption, which will generate a higher growth performance (Dawood *et al.* 2015).

In general, the organic acids are among the most promising substances as they have been reported to possess anti-*Vibrio* spp. activities (Mine and Boopathy 2011; Adams and Boopathy 2013; da Silva *et al.* 2013), and increased survival rate of shrimps (Walla *et al.* 2012; Su *et al.* 2014; Romano *et al.* 2015; Ng *et al.* 2015). Astaxanthin, a type of carotenoid, can also improve shrimp survival rate and enhance resistance to several stress conditions, such as low dissolved oxygen, low salinity, low temperature, and ammonia stress (Flores *et al.* 2007; Niu *et al.* 2009). Therefore, both organic acids and astaxanthin have the potential to be used in shrimp farming as feed additives. The objectives of this study were to evaluate the effect of dietary supplementation of formic acid and astaxanthin on growth, survival and tolerance to *V. parahaemolyticus* infection in Pacific white shrimp under laboratory conditions.

Marine Immunostimulats

Immunostimulants are substances, which elicit non-specific defense mechanisms and enhance the barrier of infections against pathogens. They are isolated from natural sources and then synthesised chemically (Example: cell wall preparations from bacteria, fungi, mushroom). Most of the research on immunostimulants has been directed towards treatment of cancer in humans. Immunostimulating compounds induced production of cytokine proteins like interleukins, interferon, tumor necrosis factor and colony stimulating factors.

Wang *et al.* (2017) have recently reviewed the current knowledge and future perspectives about the application of immunostimulants in aquaculture. The active principles of immunostimulatory cell wall preparations are various muramylpeptide fragments, lipopolysaccharides, lipopeptides, acyloligopeptides and specific ides composed of glucose units which are linked through β -1, 3 and β -1, 6 bonds. These glucans can exist in various structural forms, water soluble oligomers; water insoluble macromolecules and particulate matters. Mary Jane *et al.* (2015) gave an updated account about the uses of immunostimulants in shrimp culture. In shrimps so far, 11 types of pattern recognition receptors (PRRs) have been identified viz., β -1,3-glucanase-related proteins, β -1,3-glucan-

binding proteins, c-type lectins, scavenger receptors, galectins, fibrinogen-related proteins, thioester-containing Down syndrome cell adhesion molecules, serine protease homologs, trans-activation response RNA-binding protein, and Toll-like receptors. Aside from pattern recognition, these PRRs have different binding specificities and effector functions (Wang & Wang 2013).

In fishes, the killed mycobacteria and muramyl dipeptide enhanced resistance of coho salmon, *Oncorhynchus kisutch* against several bacterial pathogens. Injection of the synthetic lactoyl tetra peptide FK-565, increased the phagocytic activity and non-specific resistance of rainbow trout against *Aeromonas salmonicida* infections. Resistance of carp to infections by *Edwardsiella tarda* by activating the non-specific was achieved by administration of schizophyllan, scleroglucan and lentinar. The non-specific disease resistance in Atlantic salmon was enhanced by glucan preparation from *Saccharomyces cerevisiae*. Since then several researchers have suggested the possible use of glucans against viral infections in fish and shrimps.

In crustaceans, immunostimulants can increase the phagocytosis of pathogens by activating phagocytic cells in the hemolymph, increase the antibacterial and antiseptic properties of hemolymph, activate the prophenoloxidase system and mediate signal recognition and phagocytosis (Wang *et al.* 2017). In shrimps, the Wheatgerm Agglutinin (WGA), a lectin, administered as feed additive has promoted the bacterial resistance of *Penaeus orientalis*. M-Glucan (a mixture of insoluble β -1, 3 and β -1, 6 poly glucoses) was found as a short-term immunostimulant for the shrimp, *Penaeus monodon*. Immersion treatment with yeast beta-glucan was demonstrated to enhance growth and vibriosis resistance in tiger shrimp *P. monodon*. In the treated shrimps, the disease resistance could be correlated with enhanced phenoloxidase activity and intrahemocytic production of superoxide anion. In shrimps the prophenoloxidase ('Propo'), the defense enzyme system, is activated by immunostimulants. The activation of 'Propo' results in recognising pathogens and providing resistance.

In fish, the non-specific defense system is activated by the immunostimulants. The first

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line of defense - i.e., non specific humoral defense or proteases, lysins and agglutinins in mucous cell secretion; The second line of defense provided by the mucosal lining cells and the third line of defense achieved by blood cells, especially granulocytes and monocytes which destroy microbes present in the circulation are activated. Endocytically active cells such as endothelial cells, macrophages and granulocytes in organs and tissues, which degrade microbes or microbial products, take up the final defence. The final endocytic and degradation process strongly depend on the effectiveness of reticulo endothelial system, which consist of endothelial cells, and macrophages, which line the small blood vessels (sinusoids and ellipsoids). The central cells in the production of antimicrobial substances are macrophages and granulocytes, which are activated by the immune enhancers.

Hemocytes are also activated by immunostimulants. In addition, they enhance the clotting activities and produce bactericidins. In tiger shrimp *Penaeus monodon*, increased bacterial clearance was noted after injection with glucan. The bacterial clearance ability of haemolymph drawn from the tiger shrimp *Penaeus monodon* immersed in a viable cell suspension of *Vibrio vulnificus* showed that *Vibrio* cells were largely eliminated from shrimp haemolymph within 12 h following invasion and completely undetectable at 24 h. The anti-*E. coli* activity of plasma, phenoloxidase (PO) activity, as well as the production of superoxide anion (O_2^-) were significantly enhanced due to administration of glucan and zymosan. Immunostimulants can promote recovery from the status of immunosuppression caused by stress. The peptidoglycan- fed black tiger shrimp exhibited a higher tolerance to dissolved oxygen, salinity and stress than those fed with the controlled diet.

The immunostimulants have several advantages:

1. Being natural products, there is no environmental hazard.
2. Unlike vaccines, which give protection to a specific pathogen, immunostimulants provide a wide range of protection against several pathogens.
3. Most of the immunostimulants can be synthesized and the problem of residual effect on shrimps or fish is not encountered.
4. Shrimps depend more heavily on non-specific defense mechanisms than mammals and therefore immunostimulants have a significant role in health management

strategies in aquaculture.

5. When glucans were administered along with *Aeromonas hydrophila* vaccine, the response was even more enhanced, suggesting that yeast glucans have important role in disease management in warm water aquaculture.

In shrimps, three main types of circulating haemocytes have been identified and isolated by isopycnic centrifugation on Percoll gradient. Semi granular cells respond to microbial polysaccharides such as lipopolysaccharides and B-1,3-glucans by degranulation. Since the degranulated cells attach and spread on foreign surfaces, they have an important role in encapsulation. Granular haemocytes with large granules are a repository for the prophenoloxidase (pro-PO) activating system. In crustaceans, clotting is mediated by coagulogens present in the plasma and also compartmentalized within circulating cells. The plasma factor is converted to covalently linked polymers of coagulins by Ca^{2+} -dependent transaminase whereas the cell factor is converted to a gel by a serine protease proclotting enzyme, which may be triggered, by microbial molecules such as lipopolysaccharide (LPS) and -1,3-glucans.

Table Marine organisms with promising immunostimulatory effect

Source organism	Experimental organism	Assay/inhibitory activity
<i>Porphyra yezoensis</i> (Macroalga)	Murine	Phagocytic assay
<i>Undaria pinnaftifida</i> (Macroalga)	NS	Phagocytic assay
<i>Ecteinascida turbinata</i> (Tunicate)	Eel	Phagocytic assay
<i>Haliotis discus hannai</i> (Abalone)	Trout	NK cell assay
<i>Hyrtiss erecta</i> (Sponge)	NS	Immunomodulatory
<i>Briareum exavatum</i> (Gorgonid)	NS	Immunostimulatory
<i>Ulva fasciata</i> (Seaweed)	Shrimp and Fish	Immunostimulatory

NS – Not Specified

Shrimp aquaculture though lucrative, has its own threats. The sustainability of shrimp farming is achieved by a comprehensive and holistic way of managing the adverse conditions with better feeding and disease management strategies. In this context, certainly, the marine metabolites are of immense use and they are to be explored further.

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Evaluación de la Atractabilidad, Palatabilidad y Consumo de Ingredientes en Alimentos Balanceados para el Camarón Blanco del Pacífico

Litopenaeus vannamei

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Resumen

Un total de 17 ingredientes fueron evaluados en términos de atractabilidad, palatabilidad y consumo de alimento con la finalidad de incorporarlos en alimentos balanceados para camarón blanco del Pacífico *Litopenaeus vannamei* producidos por compañías de piensos en América Latina. Como dieta de referencia (DR) se utilizó una dieta comercial mexicana, la cual se molió, se le adiciono 1% alginato de sodio como ligante y se agregó el ingrediente a evaluar ya sea al 3% o 5% según fue el caso; posteriormente fue reprocesada en un molino de carne para elaborar las dietas experimentales. Se utilizaron 20 juveniles tardíos *Litopenaeus vannamei* en acuarios de 120 L en un sistema de recirculación de agua marina sintética. Los ingredientes más atractantes, palatables y mejor consumidos por los camarones fueron BioKrill, y H mix squid, es importante mencionar que la adición de cualquiera de los ingredientes mejoró la atractabilidad, palatabilidad y consumo de alimentos que de la dieta de referencia.

Palabras Clave: atractabilidad, palatabilidad, consumo de alimento, *Litopenaeus vannamei*

Abstract

Seventeen ingredients were evaluated in terms of attractiveness; palatability and feed intake with the purpose of incorporate them in aqua feeds for Pacific white shrimp *Litopenaeus vannamei* produced for feedstuffs companies in Latin America. As a reference diet (DR) a Mexican commercial diet was use, which was ground, 1% sodium alginate was add as a binder and the ingredient to be evaluate was added to either 3% or 5% as appropriate; subsequently reprocessed in a meat mill to prepare the experimental diets. Twenty late juveniles *Litopenaeus vannamei* were use in 120 L aquaria in a synthetic seawater recirculation system. The most attractive, palatable and best consumed ingredients for shrimp were BioKrill, and H mix squid, it is important to mention that the addition of any of the ingredients improved the attractiveness, palatability and feed intake of reference diet.

Keywords: attractability, palatability, feed intake, *Litopenaeus vannamei*

Introducción

Los camarones marinos poseen el sentido de la vista muy pobre, así que los animales para poder reconocer su habitat requieren de otros sentidos como el tacto (quimiorreceptores localizados en el flagelo antenal), lo que les permite encontrar el alimento (atractabilidad) y adicionalmente, distinguir entre un alimento suave o duro. El sentido del gusto (quimiorreceptores ubicados en las quelas de los pleopodos, maxilípedos y región bucal) es el responsable de seleccionar el alimento para ser ingerido (palatabilidad) y el sentido olfatorio (quimiorreceptores localizados en las anténulas). Los quimiorreceptores están íntimamente relacionados con la atractabilidad y palatabilidad de los alimentos. Estos quimiorreceptores pueden distinguir entre muchos tipos de químicos, lo que permite a los camarones seleccionar entre alimentos comestibles de otros rechazables, así como el tiempo que los animales permanecerán cerca del alimento. Los ingredientes quimio-estimulantes son aquellos que mejoran el consumo de un alimento afectando alguna de las fases del comportamiento alimenticio como la excitación, iniciación de búsqueda, localización de alimento, palatabilidad y consumo. Se considera como ingrediente atractante aquellos que poseen grandes cantidades de metabolitos de bajo peso molecular solubles en agua, que son incluidos en las dietas para camarón con la intención de atraer a los animales y promover el consumo de la dieta. Los ingredientes de origen acuático marino son excelentes fuentes atractantes y mejoran la palatabilidad y promueven el consumo de alimento. Existe una tendencia de mejorar la velocidad del consumo de alimento, en gran medida debido al serio problema de enfermedades en etapas tempranas de crecimiento; existen trabajos previos que investigaron el uso de aditivos a base de animales marinos para mejorar el rendimiento de camarones donde se demuestra que pueden mejorar el rendimiento, asociado a un crecimiento más rápido: mejorando la atractabilidad, estimulando fases apetitivas de la conducta tales como excitación, iniciación de la búsqueda, y localizar la comida; y mejorar la palatabilidad y, por lo tanto, aumentar consumo (Lee & Meyers, 1997; Samocha *et al.* 2004; Sanchez *et al.* 2005; Smith *et al.* 2005; Nunes *et al.* 2006; Suresh *et al.* 2011).

C.D. Dervy *et al.* (2016) encontraron que la harina de krill aumenta la tasa de alimentación en camarones. Esto ocurrió cuando el nivel de inclusión de

la harina de krill era de 6%. Ellos asocian que la harina de krill contiene moléculas tales como aminoácidos libres, aminos biogénicas, nucleosidos, nucleótidos, péptidos y ácidos orgánicos que actúan como quimio-atractivos para los camarones.

Estudios recientes en Facultad de Ciencias Biológicas, Programa Maricultura, UANL.

Los ingredientes experimentales a evaluar en alimentos para camarón blanco *L. vannamei* fueron proporcionados por la compañía Sudamericana, los ingredientes evaluados fueron los siguientes: 1.- Harina Krill (atk), 2.- Harina de Calamar (Squid north), 3.- Harina de Krill (Krill mix), 4.- Harina de Calamar (Squid south mix), 5.- Harina de Krill (Biokrill), 6.- Peptonas de Calamar, 7.- Harina de Krill baja en grasa (Krill low fat), 8.- Harina de Calamar (Squid south), 9.- Harina de Langostino (Lango meal sic), 10.- Hidrolizado de calamar (H mix squid a un nivel 3%), 11.- Hidrolizado de crustáceos (H mix Crust a un nivel 3%), 12.- Hidrolizado de calamar y pescado (H mix squid/fish a un nivel 3%), 13.- Soluble de pescado (Nivel 3%), 14.- Hidrolizado de calamar (H mix squid a un nivel 5%), 15.- Hidrolizado de crustáceos (H mix Crust a un nivel 5%), 16.- Hidrolizado de calamar y pescado (H mix squid/fish a un nivel 5%), 17.- Soluble de pescado (Nivel 5%) y 18.- Alimento comercial para camarón.

Análisis químicos de ingredientes

La composición bromatológica en los ingredientes experimentales fue determinada en el laboratorio de Maricultura: Humedad, proteína cruda, lípidos, ceniza y fibra (AOAC, 1997). Extracto libre de nitrógeno (ELN) fue calculado por diferencia.

Formulación y elaboración de las dietas

Selección de alimento de referencia

Con la finalidad de contar con valores de referencia (atractividad y palatabilidad), obtenidos en nuestras instalaciones para una dieta comercial típica utilizada en granjas mexicanas, se realizó una prueba preliminar evaluando dos dietas comerciales mexicanas para camarón

(proporcionadas por el Programa Maricultura), el protocolo que se utilizó para esta prueba se describe más adelante. La dieta que presento los valores más bajos de atractabilidad y palatabilidad fue seleccionada para ser considerada como el alimento de referencia, ya que esta podría ser mejorada.

Alimentos experimentales

Para la evaluación de la atractabilidad, palatabilidad y consumo que conferirían cada uno de los diferentes ingredientes a la dieta, se formularon y elaboraron 9 alimentos experimentales y un alimento o dieta de referencia (Tabla 1). Como dieta de referencia (DR) se utilizó una dieta comercial seleccionada anteriormente, la cual se molió, se le adiciono 1% alginato de sodio como ligante y 3% del ingrediente a prueba y posteriormente fue reprocesada en un molino de carne (Torrey®). La formulación de las dietas experimentales se presenta en la Tabla 1.

Para la elaboración de las dietas experimentales los ingredientes fueron mezclados durante 15 min en una batidora (Kitchen Aid®), posteriormente se agregó 350 mL de agua tibia y se continuó mezclando por 5 min. La mezcla fue procesada en un molino de carne (Torrey®) utilizando un dado con una configuración de 1.8 mm de diámetro, la temperatura al comienzo del proceso fue de 65 ° C y al final de 95 ° C, el total de la mezcla (1 kg fue procesada en 20 minutos. Posteriormente los fideos fueron secados en un horno de convección a 130 ° C durante 8 minutos, después se dejaron a temperatura ambiente durante 12 horas y finalmente empacadas en bolsas con cierre.

Tabla 1.-Formulas experimentales

	DR	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17
Dieta Comercial Molida	990	960	960	960	960	960	960	960	960	960	960	960	960	960	940	940	940	940
Alginato de sodio	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Harina 1	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Harina 2	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Harina 3	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Harina 4	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-
Harina 5	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-
Harina 6	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-
Harina 7	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-
Harina 8	-	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-
Harina 9	-	-	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-
Hidrolizado 1 Nivel 1	-	-	-	-	-	-	-	-	-	-	30	-	-	-	-	-	-	-
Hidrolizado 2 Nivel 1	-	-	-	-	-	-	-	-	-	-	-	30	-	-	-	-	-	-
Hidrolizado 3 Nivel 1	-	-	-	-	-	-	-	-	-	-	-	-	30	-	-	-	-	-
Hidrolizado 4 Nivel 1	-	-	-	-	-	-	-	-	-	-	-	-	-	30	-	-	-	-
Hidrolizado 1 Nivel 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	-	-	-
Hidrolizado 2 Nivel 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	-	-
Hidrolizado 3 Nivel 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	-
Hidrolizado 4 Nivel 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

Análisis bromatológico de alimentos

La composición química en las dietas fue determinada utilizando la misma metodología descrita en la sección de ingredientes experimentales.

Análisis de hidroestabilidad y absorción de agua de los alimentos experimentales

La pérdida de materia seca, proteína y absorción de agua fue determinada en las dietas experimentales (Villarreal-Cavazos, 2014).

Bioensayos

Bioensayo de atractabilidad y palatabilidad

Animales.

Se utilizaron 20 camarones (*Litopenaeus vannamei*) de 6.6 g de peso promedio por cada acuario (120 L), los camarones fueron enviados de la compañía FITMAR, S.A. de C.V. ubicada en Mazatlán, Sinaloa, México.

Sala de Bioensayos

Se utilizaron acuarios de 120 L de capacidad, hechos de fibra de vidrio con un sistema de recirculación interna del agua ("air-water lift"). Además, cada acuario recibió un flujo constante de 700 mL por minuto de agua marina del sistema de recirculación, los parámetros de calidad de agua fueron medidos semanalmente (temperatura, salinidad, pH, nitratos, nitritos, nitrógeno amoniacal total). El agua marina utilizada fue agua marina sintética.

Diseño experimental.

Se trabajó con 5 replicados (5 acuarios) para evaluar dos dietas por cada acuario utilizando una distribución completamente al azar. Se realizaron dos evaluaciones al día, la primera a las 9:00 y la segunda a las 14:00 hrs.

Alimentación

En cada acuario se ofrecieron dos dietas experimentales diferentes (20 pellets de cada una), colocándolas en el piso del tanque en lugares separados (esquinas frontales del acuario) y se consideró como tiempo cero el momento en que las dietas estaban ya en el fondo del tanque, inmediatamente después se inicia la medición del tiempo de arribo, que es el tiempo que se tardaban los animales en llegar a cada una de las dietas, la medición se realizó para cada dieta por separado mediante la utilización de un cronómetro digital: así mismo, se mide el tiempo total que es el tiempo que los camarones tardan en llevarse la totalidad del alimento (20 pellets). El tiempo que transcurre desde que las dietas experimentales son ofrecidas a los animales hasta que estos se llevan la totalidad de los pellets ofrecidos lo consideramos como palatabilidad (tiempo total).

Análisis estadístico.

Se realizó un análisis bi-factorial considerando como primer factor el tratamiento (dieta) y como segundo factor el horario de alimentación (am/pm), posteriormente se realizó una comparación múltiple de medias (Prueba de Tukey) para establecer diferencias entre los tratamientos, utilizando el paquete estadístico SPSS versión 16.

Bioensayo de consumo de alimento

Animales

Se utilizaron 600 camarones (*Litopenaeus vannamei*) de 6.8 g de peso promedio, los camarones fueron enviados de la compañía FITMAR, S.A. de C.V. ubicada en Mazatlán, Sinaloa, México.

Villareal-Cavazos, D. et al., 2017. Evaluación de la Atractabilidad, Palatabilidad y Consumo de Ingredientes en Alimentos Balanceados para el Camarón Blanco del Pacífico *Litopenaeus vannamei*. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villareal-Cavazos, D. A., Gamboa-Delgado, J., López Acuña, L.M. y Galaviz-Espinoza, M. (Eds), Investigación y Desarrollo en Nutrición Acuícola Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, pp. 523-540. ISBN 978-607-27-0822-8.

Sala de Bioensayos

Se utilizaron dos acuarios de 500 L de capacidad, hecho de fibra de vidrio con un sistema de recirculación interna del agua ("air-water lift"), recibió un flujo constante de 2,130 mL por minuto de agua marina del sistema de recirculación, los parámetros de calidad de agua fueron medidos semanalmente (temperatura, salinidad, pH, nitratos, nitritos, nitrógeno amoniacal total).

Alimentación

Comederos

Se construyeron comederos de PVC hidráulico de 6'' de diámetro x 1'' de altura, con un fondo metálico de malla de acero inoxidable (200 μm), soldados por calor. Los animales fueron entrenados o adaptados ofreciéndoles el alimento únicamente en los comederos durante 5 días previos al inicio de la prueba (Imagen 1).

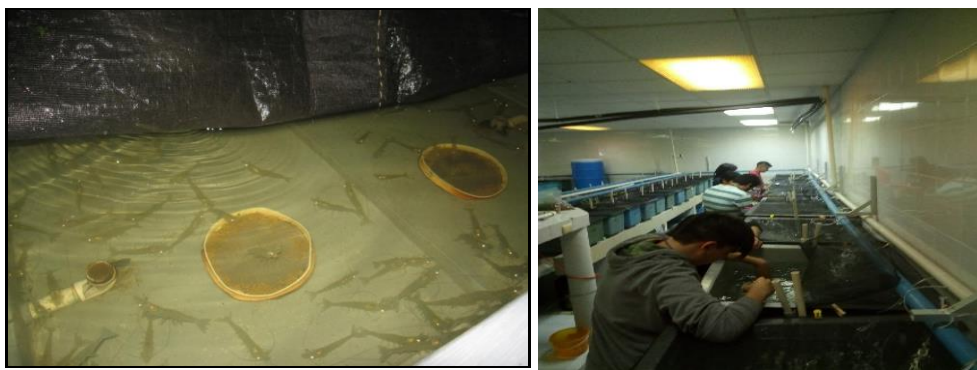


Imagen 1.- Camarones siendo adaptados a los comederos.

Raciones alimenticias durante la prueba

Se ofreció alimento *ad libitum* dos veces al día, la primera ración fue a las 8:00 horas y la segunda ración a las 14:00 horas. Los comederos fueron colocados siempre en la misma posición en el tanque (cerca de las esquinas a 30 cm de cada pared y a 50 cm de distancia entre cada comedero). Se ofrecieron 4 dietas por día (siempre la dieta de referencia vs 3 dietas

experimentales por día) y las repeticiones (tres por cada dieta experimental) fueron realizadas a través del tiempo. Los tratamientos se ofrecieron de forma alterna en días diferentes. Todo el alimento fue ofrecido en comederos previamente tarados y llenados con 8 gramos de alimento por ración (cada comedero con una dieta diferente) y ofrecidos a los animales durante 1 hora, posteriormente eran retirados y puestos a secar a 65⁰ C durante 3 horas en un horno de convección y finalmente pesados utilizando la siguiente fórmula para el cálculo de consumo de alimento:

$$\text{Consumo alimento} = [(\text{peso del comedero} + \text{alimento húmedo al inicio}) * (\% \text{ MS})] - [\text{peso del comedero} + \text{restos de alimento seco al final}] / \text{número de camarones.}$$

Adicionalmente se calculó el consumo de alimento relativo a la dieta de referencia (CRDR), el cual fue obtenido de la siguiente manera: $\text{CRDR} = (\text{alimento consumido/DR}) * 100$.

Análisis estadístico.

Se realizó un análisis bi-factorial considerando como primer factor el tratamiento (dieta) y como segundo factor el horario de alimentación (am/pm), Posteriormente se realizó una comparación múltiple de medias (Prueba de Tukey) para establecer diferencias entre los tratamientos utilizando el paquete estadístico SPSS versión 16.

Resultados

Composición proximal de los ingredientes.

El contenido de Proteína cruda (PC) de los diferentes ingredientes varió desde 50% (Lango meal sic) hasta 71% (Squid north); los lípidos está pendiente; Lango meal sic presentó el contenido más alto de cenizas 45.75% y la harina de calamar (Squid north) los contenidos más bajos (8.27%). Esta información es acorde a lo reportado por (NRC, 1983; Novus, 1996;) para productos similares. La composición química de los ingredientes experimentales se presenta en la Tabla 2.

Tabla 2.- Composición proximal de ingredientes.

	PC (%)	GC (%)	Ceniza (%)
1.- Harina de Krill (atk)	57.50	11.63	12.54
2.- Harina de Calamar (Squid north)	71.02	8.29	8.27
3.- Harina de Krill (mix)	64.39	10.55	13.25
4.- Harina de Calamar (Squid south mix)	68.38	10.01	10.05
5.- Harina de Krill (Biokrill)	57.62	8.54	24.90
6.- Peptonas de Calamar (Squid peptones)	62.64	15.61	8.82
7.- Harina de Krill bajo en grasa (Krill low fat)	62.50	7.43	14.61
8.- Harina de Calamar (Squid south)	68.99	6.11	10.76
9.- Harina de Langostino (Lango meal sic)	50.69	9.04	45.75
10.- Hidrolizado de Calamar (H mix squid, Nivel 3%)	----	----	----
11.- Hidrolizado de Crustáceo (H mix Crust, Nivel 3%)	----	----	----
12.- Hidrolizado de Calamar y Pescado (H mix squid/fish, Nivel 3%)	----	----	----
13.- Soluble de pescado (Nivel 3%)	----	----	----
14.- Hidrolizado de Calamar (H mix squid, Nivel 5%)	----	----	----
15.- Hidrolizado de Crustáceo (H mix Crust, Nivel 5%)	----	----	----
16.- Hidrolizado de Calamar y Pescado (H mix squid/fish, Nivel 5%)	----	----	----
17.- Soluble de pescado (Nivel 5%)	----	----	----
18.- Ingrediente de referencia	43.5	8.5	12.99

Composición proximal de las dietas experimentales.

El contenido de PC en las dietas experimentales oscilo entre 38.01% (dieta con hidrolizado de crustáceo mix) y 43.64%, (dieta con krill mix) mientras que la (dieta de referencia) D18 registró 43.48%. La composición química de las dietas experimentales con excepción de fibra cruda se presenta en la Tabla 3.

Tabla 3.- Composición proximal (base seca) de dietas experimentales expresado en %.

	PC	GC	FC	Ceniza
D1	42.25	8.31	6.36	12.30
D2	42.92	8.16	7.71	12.92
D3	43.64	8.30	7.36	13.88
D4	42.81	8.66	6.39	13.11
D5	42.22	8.57	6.39	13.33
D6	43.41	8.54	6.36	13.13
D7	43.01	8.22	4.92	13.16
D8	42.36	8.63	6.38	13.19
D9	42.07	8.69	6.16	14.14
D10	39.98	8.92	5.14	12.84
D11	38.08	8.78	5.36	12.71
D12	40.72	9.10	5.39	13.14
D13	40.99	8.92	5.64	12.74
D14	40.80	9.10	6.04	9.70
D15	43.60	8.65	5.38	15.14
D16	40.36	9.09	4.97	13.00
D17	43.45	8.89	5.09	13.18
D18	43.48	8.50	5.98	12.99

D1.- Harina Krill (atk), D2.- Harina de Calamar (Squid north), D3.- Harina de Krill (Krill mix), D4.- Harina de Calamar (Squid south mix), D5.- Harina de Krill (Biokrill), D6.- Peptonas de Calamar, D7.- Harina de Krill baja en grasa (Krill low fat), D8.- Harina de Calamar (Squid south), D9.- Harina de Langostino (Lango meal sic), D10.- Hidrolizado de calamar (H mix squid a un nivel 3%), D11.- Hidrolizado de crustáceos (H mix Crust a un nivel 3%), D12.- Hidrolizado de calamar y pescado (H mix squid/fish a un nivel 3%), D13.- Soluble de pescado (Nivel 3%), D14.- Hidrolizado de calamar (H mix squid a un nivel 5%), D15.- - Hidrolizado de crustáceos (H mix Crust a un nivel 5%), D16.- Hidrolizado de calamar y pescado (H mix squid/fish a un nivel 5%), D17.- Soluble de pescado (Nivel 5%) y D18.- Alimento comercial para camarón.

Absorción de agua, pérdida de materia seca y proteína cruda

La capacidad de absorción de agua en los pellets de las dietas experimentales después de una hora de inmersión en agua marina presento diferencias significativas con una $P= 0.05$ y varió entre 80.17% (D16 hidrolizado squid/fish mix 5%) y 162.00% (Krill ATK), de forma general los valores son buenos, ya que en la naturaleza los camarones se alimentan de crustáceos bivalvos, poliquetos, peces muertos que presentan un elevado contenido de agua (70-85%) y

una textura suave que les permite sostener y desgarrar el alimento (Cruz *et al.*, 2006). Únicamente la harina de Krill ATK mejoró la absorción de agua de la dieta de referencia (162% vs 142.6%), los resultados encontrados en el presente estudio se muestran en la Tabla 4.

Tabla 4.- Porcentaje de absorción de agua en dietas experimentales

	% Abs H ₂ O	% PMS
D1	162.00	13.03
D2	107.83	9.00
D3	88.44	8.90
D4	114.33	10.38
D5	114.58	6.96
D6	110.67	9.34
D7	105.08	6.80
D8	98.58	8.07
D9	94.33	6.41
D10	114.92	11.46
D11	89.17	8.49
D12	112.58	13.47
D13	107.25	10.77
D14	107.00	11.74
D15	108.67	11.01
D16	80.17	8.78
D17	109.58	13.55
D18	142.58	13.63

D1.- Harina Krill (atk), D2.- Harina de Calamar (Squid north), D3.- Harina de Krill (Krill mix), D4.- Harina de Calamar (Squid south mix), D5.- Harina de Krill (Biokrill), D6.- Peptonas de Calamar, D7.- Harina de Krill baja en grasa (Krill low fat), D8.- Harina de Calamar (Squid south), D9.- Harina de Langostino (Lango meal sic), D10.- Hidrolizado de calamar (H mix squid a un nivel 3%), D11.- Hidrolizado de crustáceos (H mix Crust a un nivel 3%), D12.- Hidrolizado de calamar y pescado (H mix squid/fish a un nivel 3%), D13.- Soluble de pescado (Nivel 3%), D14.- Hidrolizado de calamar (H mix squid a un nivel 5%), D15.- Hidrolizado de crustáceos (H mix Crust a un nivel 5%), D16.- Hidrolizado de calamar y pescado (H mix squid/fish a un nivel 5%), D17.- Soluble de pescado (Nivel 5%) y D18.- Alimento comercial para camarón.

En lo que respecta a los resultados de pérdida de materia seca, se observaron diferencias estadísticas significativas ($P= 0.05$) oscilando los valores entre 6.41% (D9, Lango Meal SIC) y 13.63% (D18, dieta de referencia). Para los resultados de pérdida de proteína cruda, están siendo

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procesados. Los valores tanto de PMS son acordes a los reportados por Cruz *et al.* (2006) para alimentos comerciales. Los resultados se presentan en la tabla 5. Las diferencias obtenidas en las dietas experimentales en términos de absorción de agua y pérdida de nutrientes son asociadas a la inclusión de los diferentes ingredientes experimentales (y su proceso de elaboración de los diferentes ingredientes). No existe un parámetro óptimo sobre la absorción de agua y pérdida de nutrientes, no obstante, entre mayor sea la absorción de agua, aunado a una pérdida de nutrientes baja son indicadores deseables en las dietas para camarón.

Atractancia y palatabilidad

En términos de atractabilidad (tiempo de arribo), todas las dietas experimentales presentaron valores más bajos que la dieta de referencia ($P= 0.05$), lo que indica que los diferentes ingredientes mejoraron el tiempo en que los camarones tardan en ser atraídos hacia a el alimento, los valores oscilaron entre 8'' (Dieta con Biokrill y Dieta con Hidrolizado de Squid mix) y 59'' (D18). No se presentó efecto asociado al horario de alimentación (am vs pm). Los resultados promedio se pueden observar en la Figura 1 en donde se aprecia que al incorporar cualquiera de los ingredientes mejoraron la atractancia de la dieta de referencia.

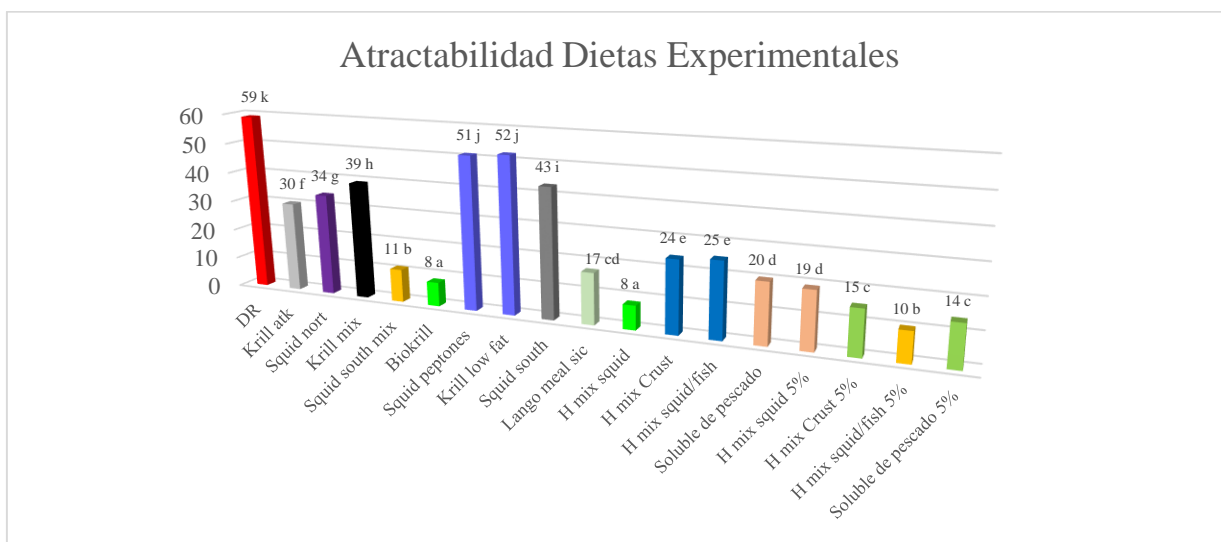


Figura 1.- Atractabilidad de las dietas experimentales expresado en segundos

DR.- dieta de referencia. H.- Hidrolizado; Letras diferentes en las barras indican diferencias significativas con una $P < 0.05$ de acuerdo a la prueba de comparación de medias Tukey.

Los ingredientes Biokrill y el Hidrolizado Squid Mix fueron los más atractantes, éste último mostró un mejor efecto atractivo al incluirlo al 3% que al 5%; adicionalmente podemos agregar que el Hidrolizados Mix Crust, Hidrolizado Fish/Squid Mix y soluble de pescado mejoran la atractabilidad de la dieta al aumentar el nivel de inclusión del 3% al 5%. Los valores generales se presentan en la Tabla 5.

Tabla 5. Valores de atractabilidad de las dietas experimentales expresada en segundos.

DR	59
Krill atk	30
Squid nort	34
Krill mix	39
Squid south mix	11
Biokrill	8
Squid peptones	51
Krill low fat	52
Squid south	43
Lango meal sic	17
H mix squid	8
H mix Crust	24
H mix squid/fish	25
Soluble de pescado	20
H mix squid 5%	19
H mix Crust 5%	15
H mix squid/fish 5%	10
Soluble de pescado 5%	14

En lo que respecta a la palatabilidad (tiempo total), los valores variaron entre 1'15'' (D4, Hidrolizado Squid South Mix) y 3'48'' (Dieta de Referencia), con una diferencia estadística significativa ($P= 0.05$). Todas las dietas experimentales presentaron valores más bajos que la Dieta de Referencia, los resultados son presentados en la Figura 2.

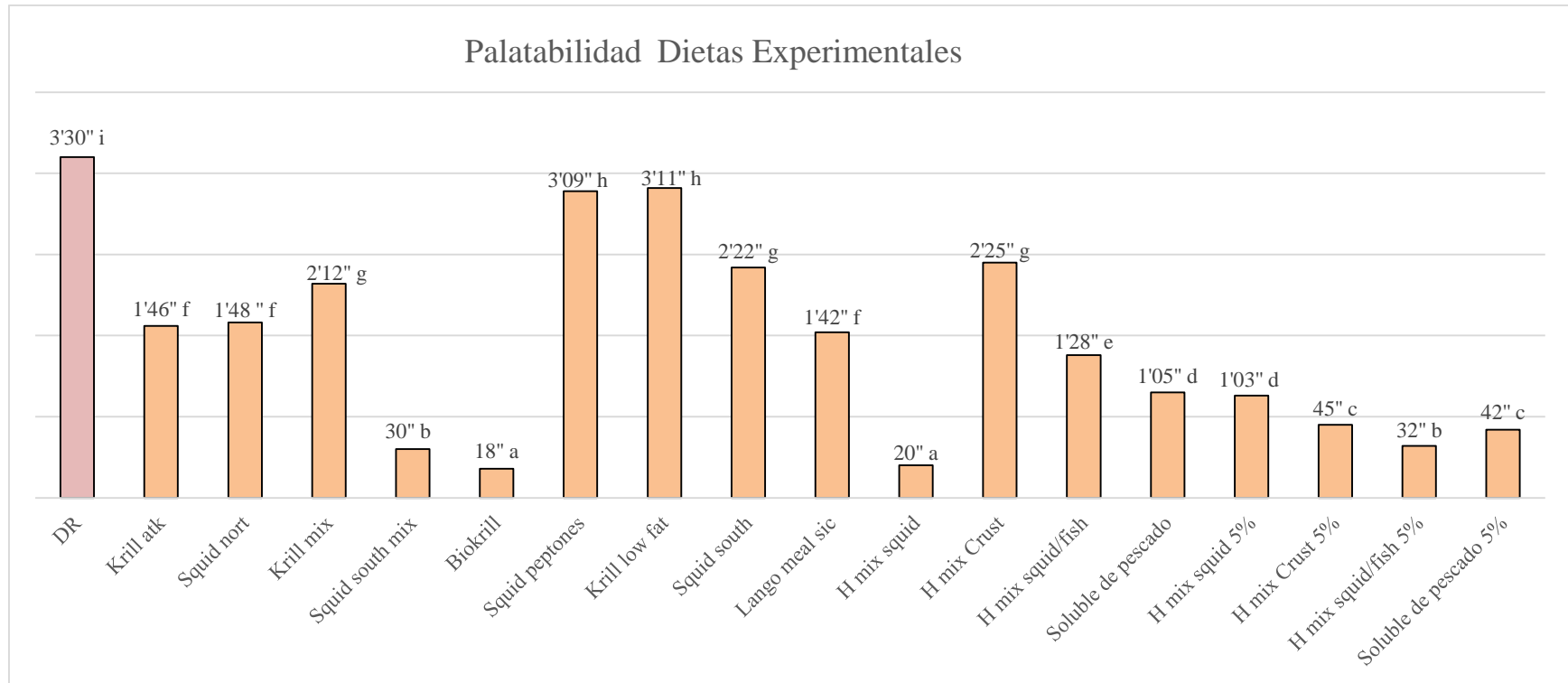


Figura 2.- Valores de palatabilidad de las dietas experimentales expresados en minutos ($P= 0.05$).

DR.- dieta de referencia (Ingrediente de referencia); H.- Hidrolizado; Letras diferentes en las barras indican diferencias significativas con una $P < 0.05$ de acuerdo a la prueba de comparación de medias Tukey.

Consumo de alimento

Todas las dietas experimentales presentaron valores de consumo más elevados que la Dieta de Referencia, se presentaron diferencias significativas en el consumo de las diferentes dietas ($P= 0.05$) pero, no se vio algún efecto del horario de alimentación sobre el consumo de alimento. Encontrándose que todas las dietas experimentales fueron más consumidas. Los valores son presentados en la Tabla 6. Los valores globales de cada replica por tratamiento son presentados en el anexo 2.

Tabla 6.- Consumo de alimento (valores promedio) expresado en gramos (%)

	Consumo am	Consumo pm	Consumo total
Dieta de Referencia	2.44 l	2.55 l	5.02 l
Dieta 1	5.22 g	5.40 g	10.62 g
Dieta 2	4.42 h	4.57 h	8.99 h
Dieta 3	3.57 i	3.61 i	7.18 i
Dieta 4	6.28 c	6.40 c	12.68 c
Dieta 5	7.60 a	7.78 a	15.38 a
Dieta 6	2.73 k	2.90 k	5.63 k
Dieta 7	2.63 k	2.70 k	5.35 k
Dieta 8	3.12 j	3.22 j	6.34 j
Dieta 9	5.09 gh	5.17 gh	10.26 gh
Dieta 10	7.61 a	7.66 a	15.27 a
Dieta 11	3.19 j	3.21 j	6.20 j
Dieta 12	5.45 f	5.55 f	11.00 f
Dieta 13	5.80 e	5.88 e	11.68 e
Dieta 14	5.71 e	5.81 e	11.52 e
Dieta 15	6.46 d	6.47 d	12.93 d
Dieta 16	6.99 b	7.04 b	14.03 b
Dieta 17	6.28 d	6.47 d	12.75 d

Letras diferentes en la misma columna indican diferencias significativas con una $P < 0.05$ de acuerdo con la prueba de comparación de medias Tukey.

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Uso profiláctico de aditivos inmunoestimulantes en el cultivo del camarón blanco, *Litopenaeus vannamei*

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Resumen

La industria del camarón representa el mayor desarrollo acuícola nacional y requiere grandes inversiones para alcanzar economías de escala y rentabilidad, debido a los problemas acentuados por el bajo margen de ganancia (15%) de los sistemas intensivos e hiperintensivos que vuelven vulnerables a los productores por la constante incidencia de enfermedades, a cambios en el mercado y en los costos de los insumos. Muchas investigaciones se han encaminado con el objetivo de prevenir o reducir la incidencia de enfermedades en los cultivos de camarón y el impacto ambiental de los productos utilizados (fertilizantes, pesticidas, herbicidas, antibióticos), lo que ha llevado a experimentar con alternativas terapéuticas naturales en el alimento y el agua de cultivo. Los aditivos como plantas medicinales, inmunoestimulantes, probióticos y prebióticos, se pueden adicionar a la dieta del camarón, por ser el medio más prometedor para influir en la salud del organismo cultivado e inducir resistencia al estrés y agentes causantes de enfermedades. Para el uso eficiente de los aditivos inmunoestimulantes en los cultivos acuícolas es indispensable el estudio del sistema inmune de las especies, debido a que es una herramienta útil para el diseño de estrategias que permiten mejorar la respuesta de defensa del hospedero contra patógenos potenciales. El presente estudio se enfoca en una revisión actualizada del uso de los diferentes aditivos inmunoestimulantes en el cultivo del camarón blanco y su eficiencia para prevenir enfermedades infecciosas de origen microbiano y viral.

Palabras clave: Inmunoestimulantes, probióticos, invertebrados, acuicultura.

Introducción

La acuicultura moderna provee de medios efectivos para la producción intensiva de organismos acuáticos bajo condiciones controladas. Tal industria, con inversión y ganancias multimillonarias, crece rápidamente (Bondad-Reantaso *et al.* 2012). El incremento de producción en granjas camaroneras con sistemas de siembra semi-intensivo e intensivos incrementa la posibilidad de introducción y transmisión de enfermedades; dado a un descontrol del crecimiento microbiano por la disponibilidad de los nutrientes y óptimos factores de crecimiento para microorganismos nocivos, además de las altas densidades de organismos cultivados causan condiciones estresantes en organismos facilitando el riesgo de epizootias en los cultivos.

La mayor amenaza para el cultivo del camarón son las diferentes enfermedades que atacan a esta especie, tales como: Síndrome de Taura (ST), Enfermedad de la Mancha Blanca (EMB), Hepatopancreatitis necrotizante (NHP), Enfermedad de la Cabeza Amarilla (YHD), Enfermedad de la Necrosis Hipodérmica y Hematopoyética Infecciosa (IHHNV), Enfermedad de la Mionecrosis Infecciosa (IMNV), pero en la actualidad siguen apareciendo nuevas enfermedades y debido al desconocimiento de éstas, causan grandes mortalidades en los cultivos tal es el caso la enfermedad de la Necrosis Hepatopancreática aguda enfermedad denominada “Síndrome de la mortalidad temprana del camarón (EMS, por sus siglas en inglés)” Conocida también como Síndrome de la necrosis aguda del hepatopáncreas del camarón (AHPNS, por sus siglas en inglés). En general, las enfermedades de cualquier índole (viral, microbiana, fisiológica, parasitaria, entre otras) que presentan los organismos cultivados, siempre han sido notadas una vez que se establecen y causan grandes pérdidas en la producción (COSAES, 2013).

La sintomatología de las enfermedades es muy similar, el organismo presenta reducción en el consumo de alimento, letargia, agotamiento, manchas (rojas, café, negras), músculo melanizado, cutícula melanizada, cambio de color en hepatopáncreas y/o atrofia; en el caso de montajes en fresco al microscopio se observa necrosis. En la presencia de epicomensales se observa un cambio de coloración en las branquias (amarillo, café, verde o negro), que depende del tipo de componente adherido a ellas. Se puede extender esta coloración a toda

la superficie del animal en casos muy severos dando apariencia de “peluche” o “algodonoso” (Cuéllar, 2013). El diagnóstico de enfermedades va más allá de la sintomatología y características físicas, incorpora también el conocimiento de su etiología, el conocimiento del agente causal, de la fisiopatología, el diagnóstico, tratamiento, pronóstico y potencialmente las ideas para minimizar, combatir y hasta erradicar una enfermedad en particular.

El uso de antibióticos en sistemas acuáticos es para mantener la salud de los organismos, sin embargo, según (Ruiz *et al.*, 2006), el uso excesivo e inapropiado de antibacterianos es el factor más importante en la aparición y diseminación de la resistencia, al crear una presión de selección que favorece la supervivencia de patógenos resistentes a los antibacterianos, también influyen la falta de diagnósticos etiológicos y de información que oriente los tratamientos empíricos y normas severas que restrinjan el uso indiscriminado de fármacos, por ende un aumento en los costos de tratamiento y en casos extremos la pérdida total del cultivo.

Uno de los factores principales para obtener un alimento acuícola de buena calidad, es la formulación de los componentes y calidad de la materia prima (Achupallas *et al.*, 2000). En la mayoría de los casos, las fuentes de Carbono/Nitrógeno (C/N) son indispensables para un óptimo crecimiento en los primeros estadios de vida donde el organismo se encuentra en un constante crecimiento por lo que la adición de proteína en la dieta debe ser elevada. Sin embargo, si la fuente de proteína de la materia prima es compleja, el organismo debe transformar estos nutrientes empleando una serie de enzimas para hacerlos biodisponibles y que puedan ser asimilados por él. El uso de microorganismos probióticos, como levaduras y bacterias, aumenta el peso y la talla del organismo que coloniza atribuyendo esta respuesta a la fermentación de los alimentos al sintetizar a las enzimas responsables de hidrólisis, como proteasas, amilasas, entre otras, ayudando en el aumento de la digestibilidad de fibras y proteínas. Hay una mejor absorción en los nutrientes formulados en el organismo, el cual gana peso y genera mayores ganancias económicas (García *et al.*, 2012).

A pesar de que hoy en día la infraestructura empleada en la acuicultura va de la mano para que los organismos tengan las condiciones óptimas para su crecimiento, tanto ambientales como nutricionales, uno de los principales retos es la calidad en la producción

de organismos acuáticos, por lo que es necesario reducir al mínimo el uso de antibióticos y reemplazarlo con el empleo de agentes con propiedades profilácticas, los cuales han demostrado ventajas significativas (Lomelí-Ortega & Martínez-Díaz, 2014) . La aplicación de levaduras y bacterias de origen marino en cultivos acuícolas aporta nutrientes y además sus derivados de paredes celulares confieren potencial probiótico con efectos que incrementan los parámetros productivos; mejoran los procesos digestivos, modulan la microbiota intestinal y el estado inmunológico. El sistema inmune del camarón reconoce y desencadena una respuesta ya sea humoral o celular ante diferentes patógenos, es por ello la importancia de mantener un estado inmunológico eficiente de los organismos en cultivo.

Cada día aparecen nuevas enfermedades que causan grandes tasas de mortandad de los organismos cultivados, para ello es necesario contar con herramientas necesarias para activar el sistema inmune de los organismos en cultivo. El interés en la prevención de enfermedades por el uso de inmunoestimulantes ha aumentado, estos alertan al sistema inmune de los organismos incrementando la respuesta a infecciones, haciendo a los organismos más resistentes (Rendón y Balcázar, 2003), diferentes componentes como lipopolisacáridos, péptidoglicanos y β -glucanos, han sido empleados como inmunoestimulantes en camarones (Vargas-Albores *et al.*, 1998; Campa-Córdova *et al.*, 2010). Los pigmentos antioxidantes como los carotenos producidos por la microalga *Dunaliella* sp. (Lomelí-Ortega & Martínez-Díaz, 2014), incrementan la resistencia de *L. vannamei* a infecciones experimentales con WSSV (Medina-Félix *et al.*, 2014). Las levaduras han sido usadas como activadores, moduladores y fortificadores del sistema inmune en diversos organismos debido a la presencia de β -glucanos, mananoligosacáridos y otros componentes de la pared celular. Esto sugiere la posibilidad de usar estos microorganismos como inmuno-estimulantes y fuente de bio-productos empleados para ese fin en acuicultura (Zhen-Ming *et al.*, 2010).

El presente trabajo se enfoca en la aplicación de agentes biológicos para estudiar la modulación del sistema inmunológico y antioxidante del camarón, con la finalidad de utilizarlos para la prevención de enfermedades y mejorar la producción en la industria acuícola.

Metodología

Cepas de microorganismos e inmunoestimulantes

Las cepas experimentales fueron obtenidas de la colección de levaduras del Centro Investigaciones Biológicas del Noroeste (CIBNOR), siendo utilizadas 8 cepas aisladas de medio marino. *Symphodiomyopsis sp.*, (D1 y N6), *Cronobacter sp.*, *Wickerhamomyces anomalus*, *Kluyveromyces aestuarii*, *Candida maris*, *Geotrichum candidum* y *Curtobacterium sp.* La microalga *Dunaliella sp.* se obtuvo del cepario del Departamento de Investigaciones Científicas y Tecnológicas (DICTUS) de la Universidad de Sonora. El β -glucano fue obtenido de *Saccharomyces cerevisiae* (Biotec Mackzymal, Tromsø), el lipopolisacárido (LPS) de la bacteria *Helicobacter coli* (Sigma, L-2630).

Preparación de los tratamientos

Las levaduras fueron cultivadas en 4% de NaCl en medio YPD (Peptona-Dextrosa para levaduras, Sigma, Y1375), con 50 μ g/ml de cloranfenicol y a 30°C durante 24 h. *Dunaliella sp.* se cultivó en medio F/8 (basado en el medio f/2 de Guillard y Ryther, 1962), que contiene una cuarta parte de nitratos y fosfatos necesarios para su óptimo crecimiento. Una vez que alcanzaron su crecimiento máximo se flocculó con sulfato de aluminio (0.15g/l). Posteriormente el concentrado de *Dunaliella sp.* se secó por liofilización para obtener el polvo y elaborar el alimento.

Preparación de patógenos

Se colectaron organismos infectados con WSSV, se tomaron porciones de músculo y se maceraron en solución salina a una relación de 1:6, para después centrifugar a 9000 g por 5 min., el sobrenadante se pasó por un filtro de membrana de 0.2 micras y el inóculo resultante se almacenó a -80 °C.

La cepa de *Vibrio parahaemolyticus* se obtuvo por donación del CIIDIR (Sinaloa, México). La bacteria se sembró en agar tripticasa de soya para su reactivación, después de 24 horas se sembraron 30 placas más de las cuales se preparó el inóculo llevado a una absorbancia de 1 y una longitud de onda de 540 nm.

Bioensayo 1. Exposición de juveniles de *L. vannamei* a levaduras vía inmersión

Se utilizaron juveniles de *L. vannamei* (14.25 ± 1.5 g) en grupos de 15 camarones en tanques de fibra de vidrio de 60 L y 28 °C. Los grupos de juveniles fueron expuestos por inmersión cada 48 h a una concentración de 1×10^6 UFC/ml de cada cepa de levadura durante 10 días: 1) Control, sin tratamientos; 2) β -glucano (0.5 mg/ml); 3) LPS (1 μ g/ml); 4) *Kluyveromyces aestuarii*; 5) *Wickerhamomyces anomalus*; 6) *Cronobacter sp.*; 7) *Candida maris*; 8) *Geotrichum candidum*; 9) *Curtobacterium sp.* Se realizó un recambio de agua de 100% a todos los tratamientos. El bioensayo se realizó por triplicado y se tomaron al azar 3 camarones por tanque para extraer hemolinfa a las 24, 48, 72 h y 216 h posteriores a iniciado el bioensayo. Se tomaron muestras de hemolinfa de cada camarón para conteo de hemocitos circulantes (CTH) y 2.0 g de tejido muscular y se almacenaron a -80°C para posteriormente determinar actividad de la enzima superóxido dismutasa (SOD).

Bioensayo 2. Exposición de juveniles de *L. vannamei* a dieta con levaduras

Se utilizaron juveniles de *L. vannamei* (14.25 ± 1.5 g) en grupos de 15 camarones en tanques de fibra de vidrio de 60 l y 28 °C. Los grupos de juveniles fueron expuestos vía oral diariamente durante 21 días a una concentración de levadura de 2% incluidas en el alimento: 1) Control, sin tratamientos; 2) β -glucano (2%); 3) *Symptodiomyopsis sp.*, cepa D1 (2%); 4) *Symptodiomyopsis sp.*, cepa N6 (2%). Se realizó un recambio de agua de 100% a todos los tanques. El bioensayo se realizó por triplicado y se tomaron al azar 3 camarones por tanque cada 7 días. Se tomaron muestras de hemolinfa de cada camarón para conteo de hemocitos circulantes (CTH).

Bioensayo 3. Exposición de juveniles de *L. vannamei* a dietas con *Dunalliella sp.* e infección con patógenos.

Se utilizaron juveniles de *L. vannamei* (6.0 ± 0.5 g) en grupos de 15 camarones en tanques de fibra de vidrio de 60 l y temperatura controlada de 28 °C por triplicado. Los grupos de juveniles fueron expuestos vía oral diariamente a dos raciones diarias de la microalga incluida en el alimento: 1) Control, sin tratamientos; 2) *Dunalliella sp* (1%); 3) *Dunalliella sp* (1.5 %);

4) *Dunalliela* sp (2%); 5) *Dunalliela* sp (2.5 %); *Dunalliela* sp (3 %). Se realizó recambio de agua diario de 100% a todos los tanques. El bioensayo se mantuvo durante 30 días y posteriormente los camarones se infectaron por alimentación forzada utilizando un inóculo de WSSV; se tomaron al azar 3 camarones por tanque para extraer muestras de hemolinfa a los tiempos 0, 24, 48, 72, 120 y 144 horas post infección (hpi). Posterior a los 30 días de administración diaria de los tratamientos, los juveniles se infectaron con *V. parahaemolyticus* y se tomaron al azar 3 camarones por tanque para extraer hemolinfa a las 48 horas post infección (hpi).

Obtención de hemolinfa

Se tomaron 100 µl de hemolinfa de la base del quinto par de pereiópodos en una jeringa para insulina, de 1 ml que contenía 400 µl de anticoagulante de citrato trisodico. Las muestras de hemolinfa se centrifugaron a 9000 g por 5 minutos para separar el plasma de los hemocitos.

Cuenta total de hemocitos

Antes de centrifugar las muestras de hemolinfa se tomaron 10 µl de cada una de las muestras y se les agregó anticoagulante al 20% de formol para su conteo al microscopio con un hematocitometro, en donde se aplica la siguiente fórmula para obtener el número de hemocitos por mililitro y se multiplicó por el factor de dilución.

$$\text{hemocitos por mL} = \frac{\text{hemocitos contados}}{\text{numero de cuadros contados}} \times 1000$$

Actividad de la superóxido dismutasa (SOD).

La actividad SOD se determinó por el método descrito por Reyes-Becerril *et al.* (2008) utilizando NBT en presencia de riboflavina. Se colocaron 10 µl v/v de hemolinfa y buffer fosfatos de potasio (50 mM, pH 7.8) con 200 µl de la mezcla de reacción (0.1 mM EDTA, 13 µM metionina, 0.75 mM NBT, 20 µM riboflavina, 50 mM buffer de fosfatos, pH 7.8). Esta solución fue expuesta a luz fluorescente (1 min) o cuando el control lograra una densidad óptica de 0.2-0.25 a 560 nm.

Cuantificación de proteína (CP)

La CP en los hemocitos provenientes de la hemolinfa (mg/mL) se determinó en un lector de placa y una prueba de micro-determinación de proteína (Bio-Rad, USDA) realizándose la lectura a una absorbancia de 595 nm (Miles *et al.*, 2001). Se efectuó una curva a partir de los reactivos estándares que posee la prueba antes señalada.

Análisis Estadísticos

Se hizo un análisis de varianza de una vía (ANOVA) usando la prueba F para analizar las diferencias entre tratamientos y controles. Los valores de $F < 0.05$ son considerados significativamente diferentes. Cuando existieron diferencias significativas, se utilizó un análisis a posteriori, usando la prueba de Tukey (HSD) para identificar la naturaleza de estas diferencias ($P < 0.05$).

Resultados

En la Figura 1 se muestran los resultados de hemocitos circulantes en juveniles tratados con microorganismos benéficos. Se observa incremento significativo ($P < 0.05$) en juveniles expuestos a LPS y *Wickerhamomyces* sp. a las 24 h de cultivo respecto al grupo control. A las 48 h, se registraron incrementos significativos en CTH en los grupos de camarones

expuestos a *C. cretensis* y *Wickerhamomyces* sp. A las 72 h se muestra incremento significativo en *C. cretensis*, *Wickerhamomyces* sp. y *Cronobacter* sp.

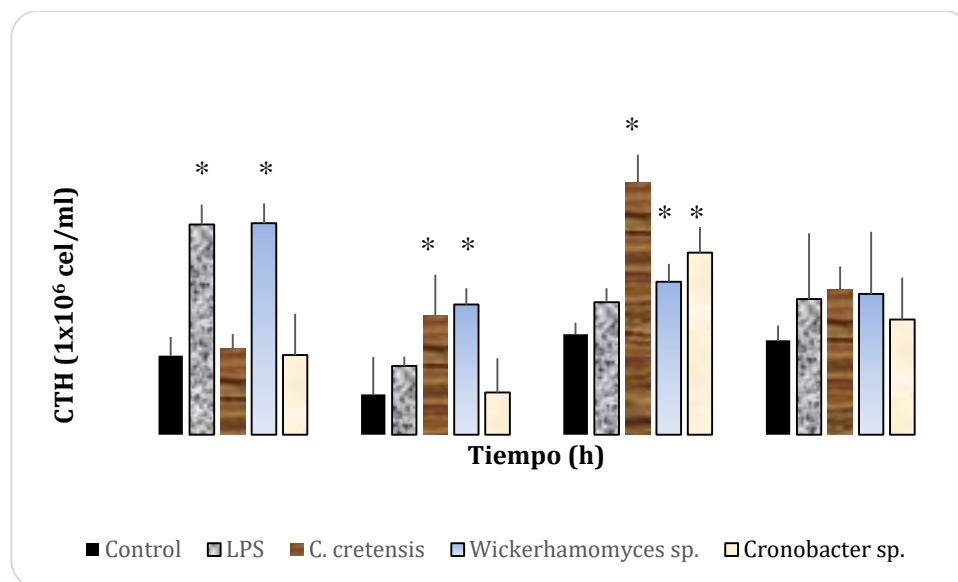


Figura 1. Hemocitos circulantes en juveniles de camarón blanco expuestos cada 48 h a una concentración de 1×10^6 UFC/ml de: 1) Lipopolisacárido (LPS; $1 \mu\text{g/ml}$); 2) *Candida cretensis*; 3) *Wickerhamomyces anomalus*; 4) *Cronobacter* sp.

(*) Significativo ($P < 0.05$) respecto al grupo control.

La actividad de la enzima superóxido dismutasa se muestra en la Figura 2. Se observa incremento significativo ($P < 0.05$) en laminarina a las 24 y 72 h posteriores al inicio del cultivo. La levadura *G. geotrichum* incrementó la actividad SOD en músculo de camarón a las 24 h y 72 h, *Candida maris* a las 48 h y *Curtobacterium* sp. a las 72 h respecto al grupo control.

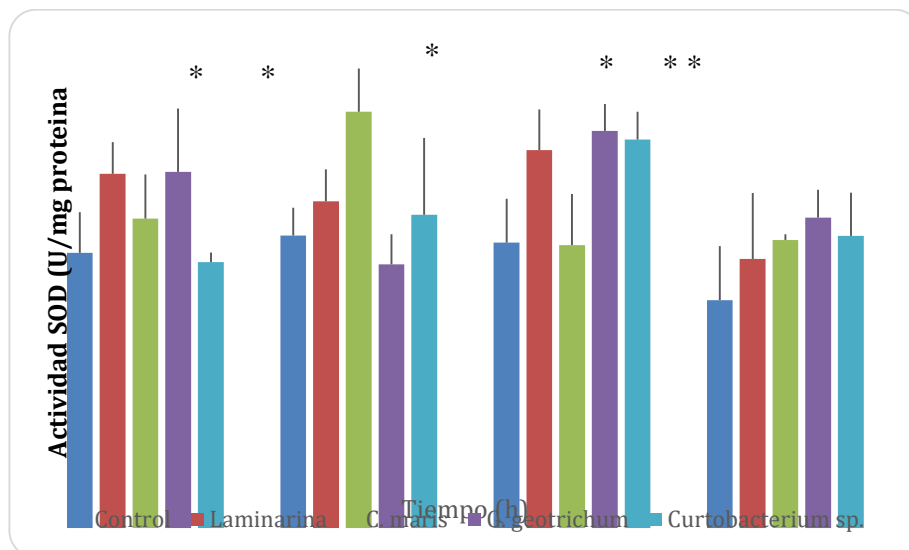


Figura 2. Actividad enzimática de superóxido dismutasa (SOD) en músculo de juveniles de camarón blanco *L. vannamei* expuestos cada 48 h una concentración de 1×10^6 UFC/ml de: 1) laminarina (0.5 mg/ml); 2) *Candida maris*; 3) *Geotrichum candidum*; 4) *Curtobacterium sp.* (*). Significativo ($P < 0.05$) respecto al grupo control.

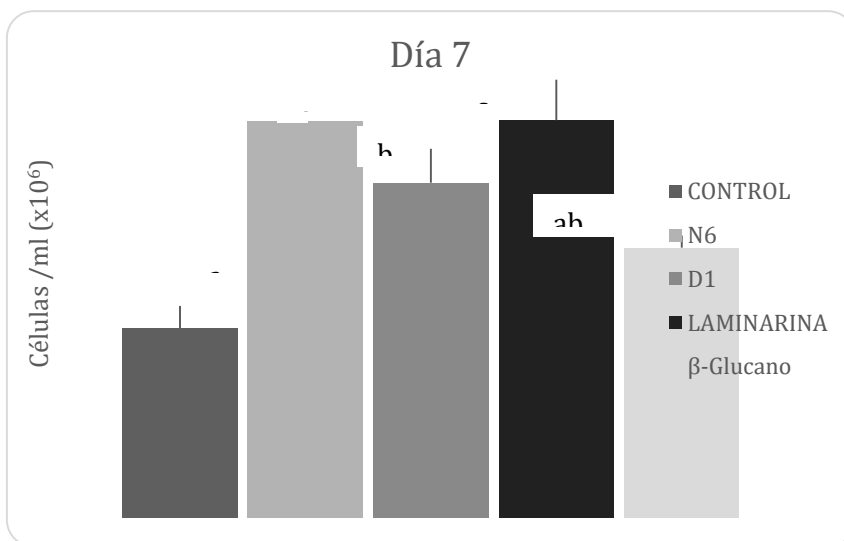


Figura 3. Concentración de hemocitos circulantes en juveniles de camarón blanco al día 7 de cultivo expuestos durante 21 días a una dieta con dos diferentes cepas de *Symphiodiomyopsis sp.*: 1) Control; 2) levadura, Cepa N6 (2%); 3) levadura, cepa D1 (2%); 4) Laminarina (0.5 mg/g); 5) β-Glucano comercial (2%).

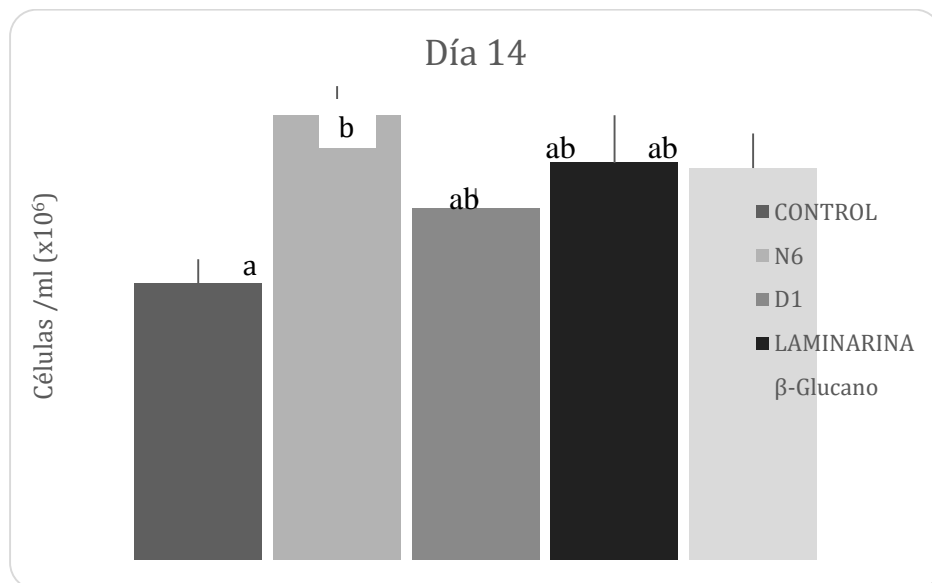


Figura 4. Concentración de hemocitos circulantes en juveniles de camarón blanco al día 14 de cultivo expuestos durante 21 días a una dieta con dos diferentes cepas de *Symptodiomyopsis sp.*: 1) Control; 2) levadura, Cepa N6 (2%); 3) levadura, cepa D1 (2%); 4) Laminarina (0.5 mg/g); 5) β-Glucano comercial (2%).

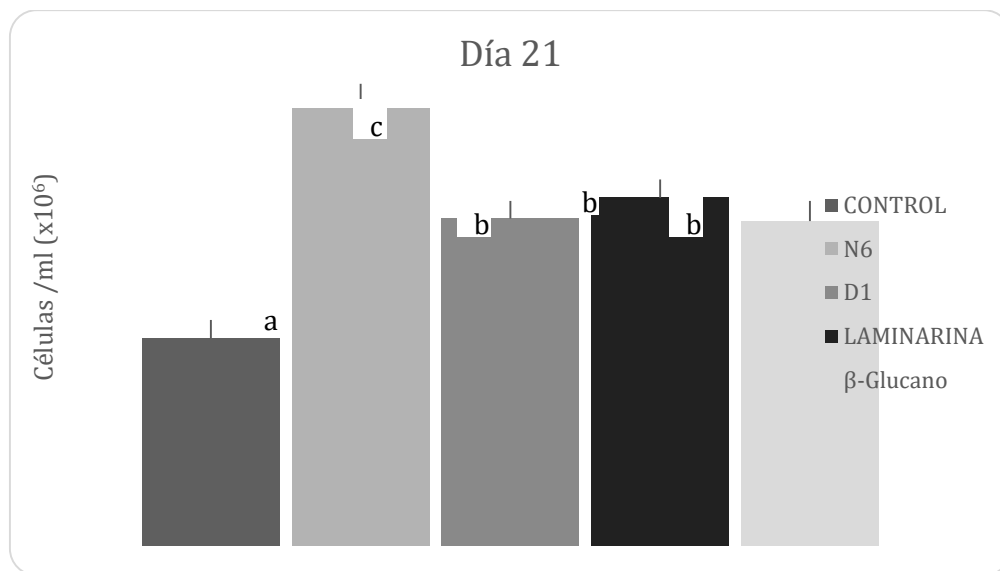


Figura 5. Concentración de hemocitos circulantes en juveniles de camarón blanco al día 21 de cultivo expuestos durante 21 días a una dieta con dos diferentes cepas de

Symphodiomyopsis sp.: 1) Control; 2) levadura, Cepa N6 (2%); 3) levadura, cepa D1 (2%); 4) Laminarina (0.5 mg/g); 5) β -Glucano comercial (2%).

La Figura 6 muestra la supervivencia de camarón alimentado con dietas conteniendo 1% y 2% de la microalga *Dunalliella sp.* e infectados con el virus de la mancha blanca. Se observa supervivencia de 83% con el 1% de *Dunaliella sp.*, seguido de la dieta con el 2% con una supervivencia de 81%, seguido de la dieta control con 56%. El grupo blanco (sin infección) no registró mortalidad.

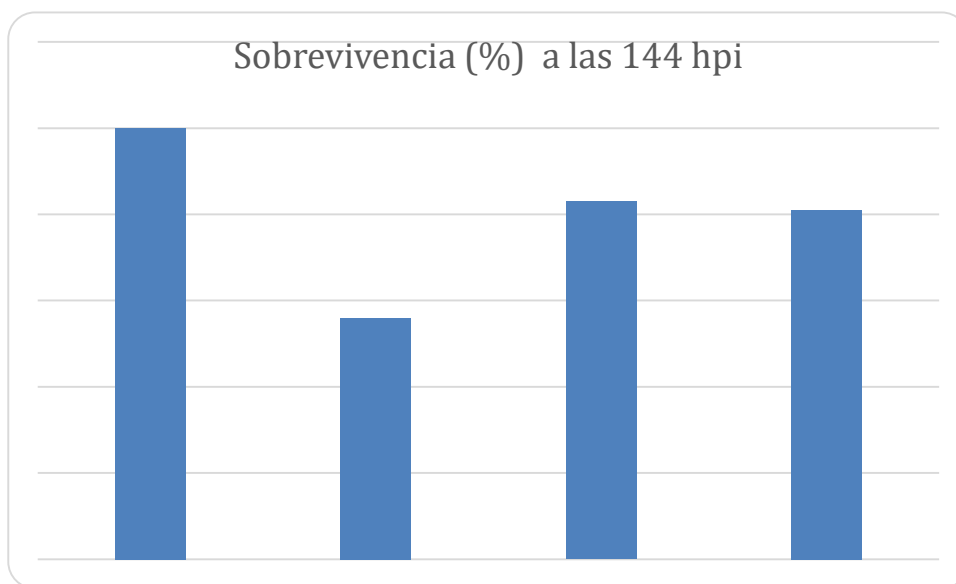


Figura 6. Supervivencia de juveniles de camarón blanco expuestos a dietas con *Dunalliella sp.* durante 30 días e infectados con WSSV.

En la Figura 7 se observa la supervivencia de camarón alimentado con dietas conteniendo 1.5, 2, 2.5 y 3% de la microalga *Dunalliella sp.* e infectados con la bacteria patógena *V. parahaemolyticus*. Se observa mayor supervivencia con la dieta al 3% (30.9%) seguida por la dieta con el 1.5% de *Dunaliella sp.* (20.9%) y una supervivencia del 13.7% en el grupo control infectado. El grupo control (sin infección) no registró mortalidad.

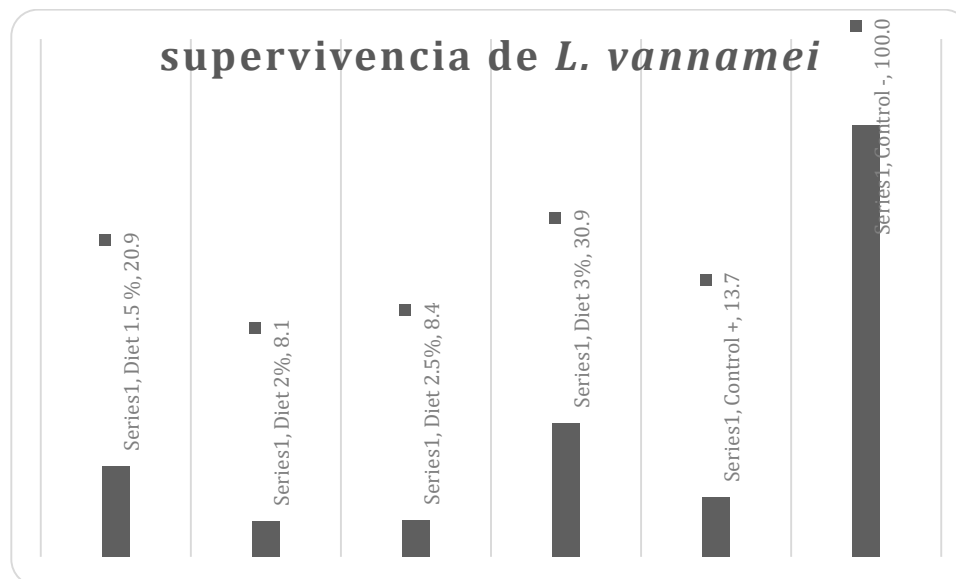


Figura 7. Supervivencia de juveniles de camarón blanco a las 48 h posteriores a la infección con *V. parahaemolyticus* expuestos previamente a dietas con *Dunalliella* sp. durante 30 días.

Discusión

Las levaduras son hongos unicelulares que han llamado la atención por su potencial para ser usados en la obtención de bio-productos tales como enzimas, toxinas, glucanos. Los inmunostimulantes por su parte son productos extraídos de las paredes de bacterias Gram negativas y/o positivas, hongos, y algas (glucanos, lipopolisacáridos, péptidoglicanos, etc.) y los cuales tienen la cualidad de activar al sistema inmune no específico (Reyes-Becerril *et al.* 2008). En la actualidad existen diversos trabajos de probióticos en acuicultura, algunos de ellos son los realizados por (Douillet y Langdon, 1994), quienes utilizaron un tipo de levadura (CA2) para incrementar la supervivencia de larvas de *Crassostrea gigas*. (Tovar *et al.*, 2000) con lubina europea (*Dicentrarchus labrax*), utilizando dos levaduras, como promotores de crecimiento (*S. cerevisiae* y *Debaryomyces hansenii*), señalan que encontraron un menor crecimiento, con las dietas que contenían ambas levaduras, pero lo atribuyen a la textura de las levaduras combinadas. Por otra parte (Reyes-Becerril *et al.*, 2008) indica que el uso de *Debaryomyces hansenii* cepa 8389 estimuló los parámetros del sistema inmune innato y específico en la cabrilla sardinera *Mycteroperca rosacea* y la dorada *Sparus aurata*.

El entendimiento de la función de los hemocitos es importante en la investigación del sistema de defensa de crustáceos, particularmente la capacidad para generar respuestas oxidativas y antioxidantes (Roch 1999) y así poder caracterizar las funciones inmunes básicas en nuevas especies de estudio. Al exponer a los camarones a diferentes cepas de microorganismos cada 48 h durante 10 días y utilizando un β -glucano y un LPS como controles positivos, se observaron tres incrementos en los hemocitos circulantes, a las 24, 48 y 72 horas después de iniciado el bioensayo. Estos incrementos en la respuesta inmune del camarón han sido reportados en otros estudios. Karunasagar *et al.* (1999) utilizó β -1,3 glucano en *P. monodon*, encontrando un pico en la producción de radicales libres de oxígeno (ROI) a las 48 horas posteriores a la administración oral. El incremento en CTH está relacionado con el estatus nutricional del camarón. Le Moullac *et al.* (1999) encontró un incremento significativo en peso alimentando al camarón con una dieta conteniendo 54% de proteína comparada con una dieta conteniendo 38%. La resistencia del camarón a la infección generada por *V. penaeicida* fue mejorada entre el 5-30%. Sajeevan *et al.* (2009) observó que las células completas de levaduras le proporcionaron a *F. indicus* un mayor CTH que el a uso de productos purificados (glucanos). Esto sugiere que las células completas pueden tener otros elementos que fortalezcan la respuesta inmune (aminoácidos, vitaminas, minerales). En el presente trabajo se observó un aumento en los hemocitos circulantes de camarones cuando estos fueron expuestos a diferentes cepas de levaduras a una dosis de 1×10^6 UFC/ml y LPS a una concentración de 1 μ g/ml. Se muestra también cómo diferentes cepas de levadura pueden activar los hemocitos en camarón a diferente tiempo de exposición.

El incremento en CTH está relacionado con el estatus nutricional del camarón. Le Moullac *et al.* (1999) encontraron un incremento significativo en peso alimentando al camarón con una dieta conteniendo 54% de proteína comparada con una dieta conteniendo 38%. La resistencia del camarón a la infección generada por *V. penaeicida* fue mejorada entre el 5-30%. La inclusión de levaduras y un β -glucano comercial en el alimento para camarón y la aplicación de una dosis diaria de 2%, incrementó la concentración de hemocitos circulantes en los camarones durante 21 días. Este estudio no explora cuánto tiempo se mantiene la respuesta inmune en camarón como respuesta a una activación con inmunoestimulantes y levaduras. Sin embargo, Sung *et al.* (1994) demostraron que la supervivencia de *P. monodon*

puede ser mejorada cuando es activada (3 horas de inmersión con glucano) 18 días antes del reto con la bacteria patógena *V. vulnificus*. En este estudio, la respuesta inmune del camarón fue activada vía inmersión y vía oral. Itami *et al.* (1998) reportaron que el uso de inmunoestimulantes por inyección, aspersion y inmersión, protegen al camarón contra infecciones experimentales. Sajeevan *et al.* (2006) siguieron que las células completas de levaduras pueden tener otros elementos que fortalezcan la respuesta inmune (aminoácidos, vitaminas, minerales) que no aportan los productos purificados (glucanos, lipopolisacáridos). Las levaduras han sido reportadas como una fuente importante de enzima SOD (Reyes-Becerril *et al.*, 2008). Las superóxido dismutasas (SODs), son una de las principales rutas de defensa antioxidante en respuesta a estrés oxidativo (Guertler *et al.*, 2010). En este estudio, la adición por inmersión cada 48 h de levaduras y laminarina (β -1,3 glucano) como control positivo (Guertler *et al.*, 2010), registraron un incremento en la actividad de la SOD en músculo de juveniles de *L. vannamei* a las 24, 48 y 72 h después de iniciado el bioensayo. Los inmunoestimulantes utilizados, incrementaron la actividad antioxidante en músculo a las 24 h posteriores a su aplicación. Guertler *et al.* (2010) señalan que la aplicación de laminarina en *Astacus astacus*, *F. paulensis*, *L. schmitti*, *L. vannamei* generó la activación de la SOD y de moléculas asociadas al sistema inmune de estos organismos. El incremento en los niveles antioxidantes en los hemocitos estimulados (dentro de las primeras 24 h), se considera una respuesta a los cambios en la composición lipídica de las membranas celulares, y de mejorar la producción de factores de activación celular como citocinas y chaperoninas, quienes pueden mejorar la capacidad fagocítica de los hemocitos (Sajeevan *et al.* 2006). Por tal motivo, se espera que el incremento en CTH y en los niveles antioxidantes en las células, como consecuencia de una exposición previa del camarón blanco a inmunoestimulantes, generen una respuesta inmune mas fuerte contra los patógenos potenciales (Downs *et al.*, 2001).

La administración de una dieta rica en carotenoides tiene un efecto protector en los camarones con una mejora en el crecimiento, reducción en la tasa de mortalidad y mejor rendimiento de los organismos, por lo tanto la administración de carotenoides es esencial para el bienestar del cultivo (Arredondo-Figueroa *et al.*, 2003). En estudios realizados por Medina-Félix *et al.* (2014) se encontró que cuando se aplica una dieta rica en antioxidantes

se obtiene una respuesta favorable por parte del camarón blanco aumentando la supervivencia en un 80%. La inclusión de *Dunaliella* sp. a la dieta de *Litopenaeus vannamei* tuvo un efecto positivo en cuanto a supervivencia, con un 83% en los camarones alimentados durante 30 días con 1% de microalga, 81% en los juveniles alimentados con 2% y el grupo control (no tratado e infectado) con el 56%. López-Elias et al. (2016) reportaron que la actividad del sistema inmune de *L. vannamei* es activado por la inclusión de *Dunaliella* sp. en el alimento de camarones, obteniendo incrementos significativos previo a una infección viral en la actividad enzimática de lisozima, aglutinina y fenoloxidasa.

La inclusión de *Dunaliella* spp. al 3% en la dieta para juveniles de *Litopenaeus vannamei* mostró una mejor supervivencia (30.9%) contra una una infección experimental con *V. parahaemolyticus*, seguida por *Dunaliella* spp. incluida al 1.5% con una supervivencia de 25.3% y el control con la menor supervivencia (10,2%). Madhumathi & Rengasamy (2011) evaluaron *Dunaliella salina* a 1 y 2% en dietas para *Penaeus monodon*, concluyendo que esta microalga es un potencial agente profiláctico contra infecciones experimentales con WSSV y por incrementar la respuesta inmune en camarón.

Los resultados obtenidos en la presente investigación sugieren que algunas cepas de levaduras, microalgas y otros géneros de microorganismos benéficos pueden ser empleadas para incrementan la proliferación de hemocitos circulantes, la actividad antioxidante y como potenciales profilácticos en juveniles de camarón blanco. Sin embargo, mayores estudios son necesarios para entender los procesos relacionados al sistema inmune de camarón con el propósito de disminuir la proliferación de enfermedades y aumentar la supervivencia y producción de camarón.

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Enteritis Inducida por la Harina de Soya en las Dietas de Peces Marinos: Efectos Sobre la Integridad del Intestino Distal y la Respuesta Inmune en *Totoaba macdonaldi*

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Resumen

El objetivo del presente estudio fue caracterizar los efectos perjudiciales en el intestino de juveniles de *Totoaba macdonaldi* producidos por el incremento en los niveles de inclusión de una mezcla de harinas de soya (SBM) en dieta en la inducción de enteritis. Cuatro dietas isoproteicas (48%) e isolipídicas (8,6%) fueron formuladas incluyendo niveles crecientes de SBM, 0%, 22%, 44% y 64% de la dieta con 1% de taurina adicionada. Al final del experimento (i.e., 8 semanas), se observó una marcada respuesta dosis-dependiente en el crecimiento producida por la inclusión de SBM. Basándose en las alteraciones histológicas del intestino distal, se observó una severa enteritis al incluir SBM por encima del 22%. Además, se observó un aumento en la exfoliación epitelial del borde de cepillo intestinal en relación con los niveles de inclusión de SBM. Los niveles de expresión de la interleucina (*il-8*) mostraron una clara respuesta inflamatoria en presencia de SBM en la dieta lo que sugiere un estado de estrés crónico que se presentan niveles más altos de inclusión de SBM (i.e., 44% y 64%). Los resultados muestran un efecto perjudicial por parte de SBM sobre la fisiología digestiva de totoaba en niveles de inclusión superiores al 22%. Por lo tanto, se sugiere que se utilice con cautela SBM al momento de formular alimentos para totoaba.

Palabras clave: *Totoaba macdonaldi*, harina de soya, expresión de interleucina, enteritis.

Abstract

The objective of the present study was to identify the detrimental effects produced in the posterior intestine in juvenile *Totoaba macdonaldi* due to increasing levels of dietary soybean meal (SBM) in the induction of enteritis. Four isoproteic (48%) isolipidic (8.6%) diets were formulated to include increasing levels of a mixture of soybean meals (SBM) at 0%, 22%, 44% 64% in a diet containing 1% taurine. At the end of the experiment (8 weeks), a marked dose-dependent response produced by SBM inclusion was observed in growth performance. Based in histological alterations in distal intestine, a severe enteritis response was found when SBM was included above 22%. Furthermore, an increase in epithelial exfoliation from the intestinal brush border was observed in relation with SBM inclusion levels. Interleukin (*il-8*) expression levels clearly showed an inflammatory response in the presence of dietary SBM suggesting a state of chronic stress in treatments containing higher levels of SBM (i.e., 44% 64%). Results indicate the disruptive effects of SBM on digestive physiology of totoaba at inclusion levels above 22%. Thus, suggesting that SBM should be cautiously used in totoaba feed formulations.

Key words: *Totoaba macdonaldi*, soybean meal, interleukin expression, enteritis.

1. Introduction

The increasing demand of seafood has led to a sustained rise in aquaculture production (FAO, 2014). Availability of raw materials for aquafeed manufacturing is one limitation towards this continued expansion. Although the so-called “reduction fisheries” that produce fishmeal (FM) fish oil are considered stable, demands for FM increases as aquaculture expands. Aquaculture annually consumes close to 70% of the FM produced worldwide, with diets for carnivorous fish species consuming the higher proportion. Therefore, the search for new protein sources to substitute FM is still of great concern in aquaculture research. Alternative vegetable protein sources, in particular soybean meal (SBM), which contains one of the highest protein levels amongst the vegetable sources, has been pointed out as one of the most promising alternative protein sources. Not surprisingly, the use of SBM has been increasingly implemented adapted into husbandry nutrition of several species over the last years. In aquaculture, defatted-SBM has been considered a viable alternative to replace at least part of FM in marine fish feeds due to high availability in the market a high crude protein content (40-48%) with a constant profile of amino acids at low cost (Gatlin *et al.* 2007). However, inclusion of high levels of SBM in carnivorous fish diets has been associated to the occurrence of intestinal enteritis (Bakke-McKellep *et al.* 2000; Krogdahl *et al.* 2003), which is defined as non-infectious inflammation of distal intestine (DI) (Baeverfjord & Krogdahl, 1996).

Additionally, SBM contains a high level of antinutritional factors for fish, apart from trypsin inhibitors (saponins, lectins, phytic acid, alkaloids, oligosaccharides, antigens), that are associated with damage of mucosal integrity, decreased pancreatic and mucosal enzymes, loss of dietary nitrogen in the faeces, thyroid hormone suppressors, lower mineral absorption, reduced palatability and suppression of the immune system (Francis *et al.* 2001; Krogdahl *et al.* 2010). Chemokines are a group of structurally related cytokines that are able to attract and activate specific types of leucocytes to the site of inflammation or injury. Therefore, the relative expression levels of interleukin (*il-8*) is a good indicator of an inflammatory reaction (Li & Yao, 2013).

Totoaba macdonaldi is the largest Sciaenidae from the Gulf of California, highlighted as an endangered species with high commercial value. Its culture is of great importance not only to stock enhancement programs, but also to stimulate its aquaculture. Under commercial conditions totoaba can reach 2-3 kg in 18 months (Juarez *et al.* 2016) and it is considered a candidate for commercial aquaculture in the Baja California region. Totoaba is a carnivorous species with a high protein demand of >50% (Rueda-López *et al.* 2011), and therefore, there is an interest to reduce dietary FM inclusion. In the present study, we evaluated the effects of increasing inclusion levels (0, 22, 44 and 64%) of a mixture of soybean meals (soy meal + soy concentrate) with a constant taurine (1%) level in the induction of enteritis in totoaba juveniles (ca. 70g) fed for 56 days and using intestinal histology and interleukin (*il-8*) expression levels as response variables.

2. Results

2.1. Distal intestine morphology

Histological analysis of the DI revealed that SBM inclusion in the diet decreased the Mucosal Fold (MF) length of the intestine with increasing coalescence of the intestinal folds (Fig. 1.B). The width of Lamina Propia (LP) and Sub-epithelial Mucosa (SM) increased with a few infiltrations of eosinophilic granulocytes with increasing content of SMB in the diet. Likewise, Goblet cells (GC) increased in number and compact groups of these structures are observed towards the apexes of MF (Fig. 1. B to D). Supranuclear Vacuoles (SV) in 22% SBM diet showed some vacuolization (Fig. 1.B) but in the 44 and 64% SBM treatments SV decreased to almost none-existing (Fig. 1. C and D). At 56 days, the same trend of shortening of the MF length with increasing inclusion of SBM in the diet was observed. Moreover, an increase width of LP, the infiltration of Eosinophilic Granulocytes (EG) into SM and LP, the coalescence of the folds and disruption of the tissue of the MF was observed with increasing dietary SMB content. Furthermore, some samples resulted in enterocyte vacuolization with high variation in vacuole size (Fig. 1. F and H). In the most severe cases, the MF was

generally shorter with a wider lamina propria and a disordered cellular arrangement (i.e., few GC and MF without the presence of SNV) (Fig. 1. G and H).

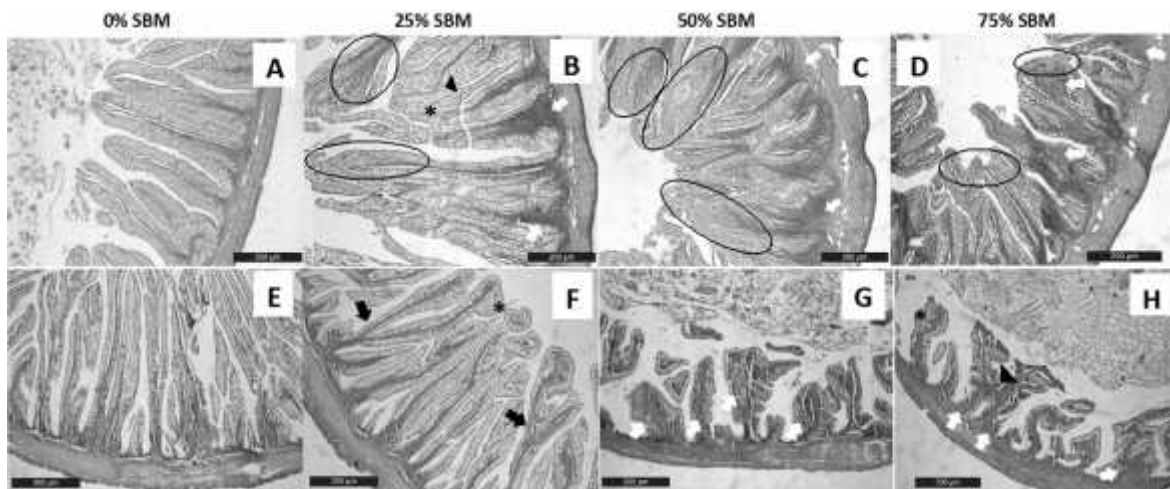


Fig.1. Histomorphological changes associated an inflammatory process of the distal intestine of *T. macdonaldi* fed diets with 0% SBM (A, E), 22% SBM (B, F), 44% SBM (C, G) and 64% SBM (D, H) during 28 days (A - D) and 56 days (E - H). Bar = 200 μ m.

2.2. Gene expression

Relative expression patterns of Interleukin (*il8*) are shown in Fig. 2. At 28 days relative *il8* expression exhibited a peak at 22% SBM diet (2.00), whereas the expression level of 0%, 44% and 64% SBM diets remained with significantly lower expression levels (0.87, 0.75, and 1.02, respectively). At 56 days, the highest expression level was found at 44% SBM diet

(1.10). Nevertheless, no significant differences were found ($P>0.05$) among SBM inclusion levels (0.93, 82.46 and 0.54 for 0%, 22% and 64%, respectively).

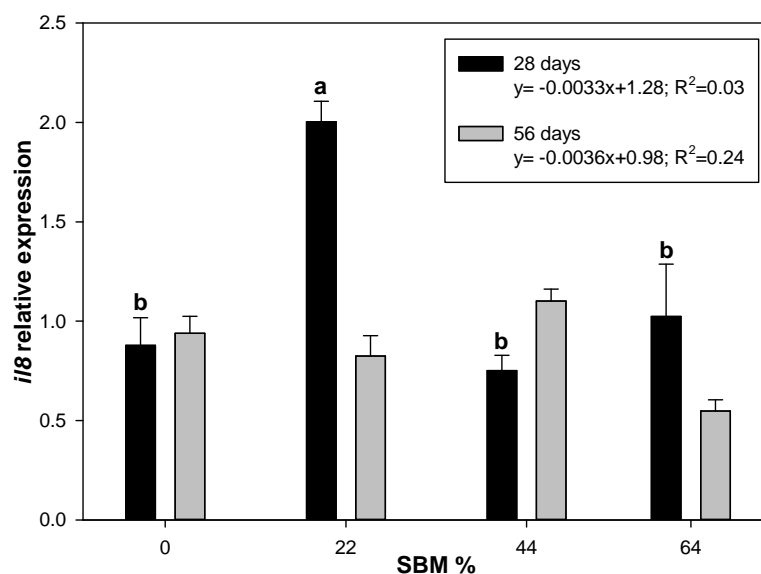


Fig. 3. Interleukin (*il8*) relative expression in *T. macdonaldi* at 28 and 56 days fed diets containing different SBM levels. Different letters represent significantly different values ($P<0.05$) within the same day ($n=9$). r^2 and equation of the regression are shown in the box.

4. Discussion

Research effort to evaluate enteritis induction by dietary alternative protein sources, including SBM, is considerable. There are plenty of reports regarding the use of SBM in carnivorous fish with contradicting results; for example some researchers confirm that SBM could be used at high levels without negative effects (Bonaldo *et al.* 2008; Trejo-Escamilla *et al.* 2016), while most reports demonstrate negative performance at intermediate or high inclusion levels (Bakke-McKellep *et al.* 2007; Chikwati *et al.* 2013; Ferrara *et al.* 2015; Gu *et al.* 2016; Urán *et al.* 2008). The present work corroborates with the majority of the literature highlighting that dietary SBM affected the overall digestive physiological performance and health status of juvenile totoaba at the intermediate or high inclusion levels.

The histological results highlighted a clear pathological detrimental inflammatory damage of the tissues analyzed (distal intestine and liver) related to dietary SBM. The integrity of the mucosal barrier is crucial to maintain tissue homeostasis against pathogens and feed antigens (Sahlmann *et al.* 2013). The mucus secreted from GC provides the first layer of intestinal protection and the integrity of mucosal barrier depends on cellular proliferation to replace damaged cells (Sahlmann *et al.* 2013).

Based on the histopathological alterations previously reported for the DI in the literature, our findings suggest that totoaba suffered a DI inflammation. This DI inflammation was dose-dependent as dietary SBM increased, resulting in a morphological change of the intestinal mucosa. The most severe cases were found in fish fed the 44 and 64% SBM diets with a clear unorganized cellular arrangement, with markedly tissue disruption, increase in coalescence of the folds and high presence of EG in the SM with migration into the LP. This is evidence of a clear process of chronic inflammation of the intestine with an important reduction of the absorption surface of the intestine reflected in the reduction of the ISI clearly documented at 56 days. These morphological changes have been suggested to be related to a cellular turnover with a concomitant increase in cell migration and apoptosis (Sahlmann *et al.* 2015).

With respect to the molecular expression levels of the immune response, interleukin-8 (*il-8*) is one of the main immune-relevant cytokines produced during an inflammatory reaction to induce wound healing during an inflammatory process (Li & Yao, 2013; Lilleeng *et al.*, 2009). As expected and in agreement with the literature, we observed an increase in *il-8* expression in fish fed SBM-based diets, including at the lower inclusion level (22% SBM), indicating a response in the posterior intestine (Bonaldo *et al.* 2015; Perera & Yúfera, 2017; Urán *et al.* 2008). The reduction in expression levels of *il-8*, demonstrate the negative effect of dietary SBM, producing a constant stress reaction, regardless of the inclusion level. Nonetheless, the reduction in expression levels, with relation to the higher SBM inclusion level, could be a consequence of the degree of damage in the DI, as verified through histological analysis. It is possible that the reduction in *il-8* expression within the DI could

be a result of the increased epithelial exfoliation from the intestinal brush border as reported for many species (Bakke-McKellep *et al.* 2007; Gu *et al.* 2016; Sahlmann *et al.* 2013).

In conclusion, the present study characterizes the limitations of SBM inclusion in *T. macdonaldi* diets, noticeably affecting the structure and physiology of distal intestine (i.e. at histological and molecular level. Additionally, our findings demonstrated a state of chronic stress in totoaba caused by high dietary SBM inclusion levels. Therefore, the present work directly suggests that SBM should be cautiously used in totoaba feeds.

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Efecto de la Dieta en el Microbioma Intestinal de Organismos Acuáticos

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Resumen

El tracto digestivo de los organismos esta colonizado por microorganismos que cumplen con variadas e importantes funciones. Mucho se ha estudiado la microbiota del tracto digestivo en organismos terrestres, pero poco en organismos acuáticos. Gracias a los recientes métodos de secuenciación masiva del ADN, se ha abierto una ventana para explorar la composición taxonómica (microbiota) de los microorganismos no cultivables que existen en todos los ecosistemas, y el intestinal no es la excepción. Además, también ha sido posible conocer los genes que están presentes (microbioma) en esos sitios al secuenciar todo el ADN presente, lo que se conoce como metagenómica.

Es ya conocido que la dieta es quizá el principal factor que puede alterar la composición tanto de la microbiota como del microbioma en animales terrestres, pero es poco lo que se conoce en animales acuáticos. Los principales factores que determinan el microbioma en peces son el nivel trófico (dieta), el hábitat y quizá su posición filogenética (Sullam *et al.*, 2012).

En el presente trabajo, se contemplarán solo los avances que se han realizado en el efecto de la dieta en la microbiota y el microbioma obtenidos por métodos de secuenciación masiva, ya que éstos están menos sujetos a desviaciones causadas por el método usado, como pueden ser especialmente los causados por métodos de cultivo dependientes o incluso aquellos cultivos independientes pero de poca resolución como la electroforesis en gel con gradientes desnaturalizantes (DGGE por sus siglas en inglés) o el análisis de conformación de una sola hebra (SSCA).

Es ya plenamente conocido que el tracto intestinal de prácticamente todos los animales estudiados contiene una alta cantidad de microorganismos comensales y una elevada diversidad taxonómica de éstos. El caso de los

organismos acuáticos no es diferente y diversos estudios se han enfocado a caracterizar la microbiota o el microbioma del tracto intestinal de peces y, en menor medida, de crustáceos.

Palabras claves: microbioma, NGS, organismos acuáticos

Peces

Microbiota núcleo

Una pregunta pocas veces realizada es si los organismos acuáticos tienen una microbiota en común, lo que se denomina “core gut microbiota” o microbiota núcleo intestinal. En peces, tanto marinos como dulceacuícolas, se ha determinado que Proteobacteria es el phylum dominante seguido de Firmicutes, Fusobacteria y Bacteroidetes, y éstos comprenden hasta el 90 % de los grupos bacterianos presentes en el tracto (Kahlke *et al.* 2012; Ingerslev *et al.* 2014; Ni *et al.* 2014; Ghanbari, Kneifel & Domig, 2015; Llewellyn *et al.* 2015; Zarkasi *et al.* 2016). En el salmón del Atlántico (*Salmo salar*) determinaron que solo 22 OTUs conformaban la microbiota núcleo de la especie (Gajardo *et al.* 2016) mientras que en la trucha arcoíris (*Oncorhynchus mykiss*) se encontraron 52 (Wong *et al.* 2013).

Debido a que estos grupos son casi siempre encontrados, se ha especulado que contribuyen de manera importante a las funciones del hospedero, tales como digestión, absorción de nutrientes y respuesta inmune y que el hábitat intestinal “selecciona” a estos grupos (Roeselers *et al.* 2011).

Microbiota residente y transitoria

Es necesario diferenciar a la microbiota residente o autóctona de la transitoria o alóctona; la primera es aquella adherida al epitelio intestinal y al mucus, y la segunda, aquella presente en las heces. En la lubina (*Dicentrarchus labrax*) encontraron que la microbiota residente era más diversa que la transitoria y lo atribuyeron a la menor presencia de bacterias asociadas al mucus en la transitoria (Carda-Diéguez, Mira and Fouz, 2014). Diferencias significativas entre ambos tipos de microbiota también se han reportado para el pargo flamenco (*Lutjanus guttatus*), con una mayor riqueza de Unidades Operacionales Taxonómicas (OTUs por sus

siglas en inglés) en la transitoria que en la residente (García, 2016). Un comportamiento similar se observó en el salmón del Atlántico (*S. salar*) en donde tanto la riqueza como la abundancia de bacterias fue mayor en la microbiota transitoria, también llamada “digesta” que en la residente o “mucosa” (Gajardo *et al.*, 2016). En la residente predominaron las Proteobacterias y en la transitoria tanto Proteobacteria como Firmicutes. El sitio de muestreo dentro del intestino también puede tener una microbiota diferente entre si, diferencias significativas se observaron entre intestino anterior e intestino medio y distal, pero no entre medio y distal en el salmón (Gajardo *et al.* 2016). En el pargo lunarejo, se obtuvieron diferencias entre taxones de bacterias en los ciegos pilóricos y en la mucosa intestinal (García, 2016).

Metanálisis han sugerido que existe una comunidad bacteriana intestinal similar entre diferente tipo de peces y que si es factible que exista la microbiota núcleo (Sullam *et al.* 2012), e incluso esta microbiota núcleo es mas similar a la presente en el intestino de vertebrados e invertebrados que a la presente en el ambiente circundante.

Factores que afectan la microbiota intestinal

Entre los principales factores que afectan la estructura taxonómica de la comunidad microbiana en el tracto intestinal de peces, se encuentran la dieta, el nivel trófico, el hábitat y probablemente la filogenia (Sullam *et al.* 2012; Miyake, Ngugi and Stingl, 2015).

Salinidad

La salinidad es un factor que tiene una influencia en la microbiota de peces, diferencias significativas han sido encontradas en la microbiota intestinal de peces de agua dulce y marina (Roeselers *et al.* 2011). Sin embargo, poco se conoce como la comunidad microbiana puede cambiar en el mismo pez cuando este es cultivado en baja o nula salinidad y luego aclimatado a una mayor salinidad o vice versa. En el caso de la tilapia del Nilo (*Oreochromis*

niloticus), la salinidad fue un factor crítico para el cambio en la microbiota intestinal (Zhang *et al.*, 2016); 55 de 89 OTUs analizadas fueron dependientes de la salinidad. Los géneros *Devosia*, *Pseudoalteromonas* y *Cetobacterium* fueron más abundantes en alta salinidad mientras que *Actinobacteria* lo fue en agua dulce (Zhang *et al.* 2016).

Sexo

Se ha visto que el sexo de algunos tipos de peces también tiene una influencia en la composición de la microbiota intestinal, tanto en peces silvestres como en cultivados (Bolnick, Snowberg, Hirsch, *et al.* 2014). Estos autores han sugerido que la inmunidad adaptativa puede constreñir a las comunidades comensales de bacterias (Bolnick, Snowberg, Caporaso, *et al.* 2014).

Inanición

La inanición es otro de los factores que afecta la composición de la microbiota; en la lubina asiática (*Lates calcifer*) notaron un significativo aumento en el número de Bacteroidetes (Xia *et al.* 2014) y se atribuye a la capacidad de este grupo de bacterias para obtener energía adicional de los alimentos. En la tilapia, también se observaron cambios en la microbiota en el colon y en el ciego en periodos de inanición (Kohl *et al.* 2014), así como en la carpa (*Ctenopharyngodon idella*) (Ni *et al.* 2014).

Dieta

Se considera que las larvas de peces son colonizadas por bacterias del agua circundante, ya que el intestino y el agua presentan taxones bacterianos en frecuencias similares (Semova *et al.* 2012). Sin embargo, la dieta (nivel trófico) modifican esta microbiota. Un análisis de varios peces y tiburones demostró que Proteobacteria era el grupo dominante en todas las

muestras, seguido de Firmicutes (Givens *et al.* 2015), pero los organismos omnívoros presentaban mayor diversidad de bacterias intestinales que los carnívoros.

La dieta es reconocida como el principal factor que influye en la diversidad y en la estructura de la comunidad microbiana en el tracto digestivo de los peces (Tarnecki *et al.* 2017). En la trucha arcoíris (*Oncorhynchus mykiss*) se encontró que la microbiota núcleo, compuesta por los phyla (Bacteroidetes, Proteobacteria, Firmicutes y Actinobacteri) no era básicamente alterada por cambios en la dieta, pero si había cambio en la microbiota “accesoria” (Desai *et al.* 2012; Wong *et al.* 2013; Ingerslev *et al.* 2014). Se ha demostrado en varios estudios que la composición de la dieta y el origen de los ingredientes pueden tener una influencia en la microbiota de la trucha, dieta con contenido vegetal tiende a aumentar la relación de bacterias del phylum Firmicutes contra las Proteobacteria cuando se compara contra una dieta a base de harina de pescado (Desai *et al.* 2012). Para el caso del pargo flamenco (*Lutjanus guttatus*), pez carnívoro, se observó un comportamiento diferente; peces alimentados con una dieta con proteína de origen vegetal y luego al cambiarle a proteína de animal marino sufrieron una considerable disminución de *Proteobacteria* (64.6%) y un aumento en *Fusobacteria* (26.25%) y *Firmicutes* (8.04%) (García, 2016) en la microbiota transitoria (heces). También se encontró que la microbiota residente del intestino distal del pargo era más susceptible a cambios en la dieta.

En el salmón del Atlántico (*Salmo salar*) se observaron cambios en la microbiota relacionados al periodo de muestreo, de una dominancia de *Bacteroidia* y *Clostridia* en meses fríos a anaerobios facultativos (bacterias ácido lácticas) en meses posteriores y no tanto respecto a la dieta (Zarkasi *et al.* 2016). Al final del experimento, el género predominante de bacterias fue *Aliivibrio*. Es importante recalcar, la amplia variación en las comunidades microbianas encontradas entre individuos de un mismo grupo (mismo tanque), lo que hace difícil determinar el efecto de un parámetro como la dieta (Zarkasi *et al.* 2016). Resultados similares en cuanto a la escasa influencia de la dieta en la microbiota fueron observados en otro estudio con el salmón (Schmidt *et al.* 2016), agrupamiento de ciertas OTUs fueron

observados entre peces alimentados con dos dietas con diferente tipo de harina, principalmente obtuvieron diferencias entre las lacto bacterias, pero también entre organismos de diferentes tanques. Truchas arcoíris (*O. mykiss*) alimentadas con dietas con harinas de pescado o de granos, mostraron ligeras diferencias en la microbiota intestinal a pesar de cambios significativos en el crecimiento de las mismas (Wong *et al.* 2013). Observaron cambios significativos en el género *Lactobacillus*, aumentando en los intestinos de los peces alimentados con dietas a base de harina de granos, mientras que *Clostridia* más abundante con harina de pescado. En la dorada (*Sparus aurata*), al igual que en los casos anteriores, la influencia de la dieta fue limitada en la riqueza de OTUs, sin embargo, hubo decremento de bacterias lácticas al sustituir harina de pescado por vegetal (Estruch *et al.*, 2015).

De estos estudios se desprende que varios factores influyen significativamente en la composición taxonómica de la microbiota de los peces y que la dieta, por lo poco que hasta ahora se sabe, no es uno de los más importantes.

Muy pocos son los trabajos que no solo analicen la microbiota, sino también el microbioma. Para el caso de la carpa herbívora (*C. idella*) observaron cambios en las rutas metabólicas de la fructuosa, manosa y piruvato, de glicerolípidos y fosfoglicerolípidos y varias de las rutas del metabolismo de aminoácidos en carpas alimentadas con centeno (Ni *et al.* 2014).

Camarón

microbiota núcleo

Para el caso de los camarones penéidos, son pocos los trabajos que investigan la microbiota de éstos organismos y menos los que relacionan la dieta con la microbiota. No se ha establecido si existe una microbiota núcleo intestinal, pero los principales phyla encontrados siempre son las Proteobacteria hasta con un 98 % del total, y en menor medida, Bacteroidetes y Firmicutes (Rungrassamee *et al.* 2013, 2014; Oetama *et al.* 2016; Wang, 2016; Vargas-Albores *et al.* 2017), este patrón se observó tanto en el camarón blanco del Pacífico (*Penaeus vannamei*) como en el tigre de Asia (*Penaeus monodon*). Sin embargo, camarones silvestres

presentan una mayor diversidad de bacterias en el intestino, pero en muchos casos, las proteobacterias y especialmente las gamaproteobacterias son dominantes (Rungrassamee *et al.* 2014; Enciso-Ibarra, 2016; Montero-Lizárraga, 2017).

No es claro cual es la relación entre los grupos bacterianos presentes en el tracto intestinal y en el agua o sedimento donde se encuentran estos camarones. Se ha observado que la comunidad bacteriana del agua difiere de la intestinal en *P. vannamei* (Johnson *et al.* 2008; Zhang *et al.* 2014; Enciso-Ibarra, 2016) lo que sugiere que el ambiente intestinal ejerce una presión selectiva sobre el establecimiento de la comunidad microbiana (Zhang *et al.* 2014).

Efecto del tipo de cultivo

Se ha observado un cambio en la microbiota intestinal de los camarones al ser cultivados, y también dependiendo del tipo de cultivo (con o sin sedimento). La diversidad alfa en el tracto digestivo disminuye al cultivar camarones silvestres (*P. vannamei*) en un sistema con sedimento, y disminuye aún mas al transferirlos a un sistema sin sedimento (Enciso-Ibarra, 2016).

Dietas

Pocos han sido los estudios del efecto de la dieta en la microbiota, y menos en el microbioma, del camarón. Camarones alimentados con un alimento balanceado (pellet) tuvieron una mayor diversidad en la microbiota intestinal que aquellos alimentados solo con calamar fresco (Enciso-Ibarra, 2016), en éstos últimos, mas del 80% de las OTUs pertenecían a proteobacterias.

Diferentes fuentes de proteína se probaron para determinar si éstas tenían un efecto en la microbiota y en el microbioma del camarón. Montero-Lizárraga (2017) preparó dietas con harina de pollo, cerdo y pescado y empleó como controles una dieta comercial de referencia y calamar crudo. Las heces provenientes de camarones alimentados con harinas de pescado,

soya y con calamar, tuvieron la menor diversidad y mayor dominancia de un grupo (Proteobacteria) y las de harina de ave y dieta de referencia, las mas diversas. Sin embargo, aunque hubo variabilidad en la estructura taxonómica de la microbiota, no se observaron mayores cambios en las rutas metabólicas del microbioma, esto es, aunque había diferentes bacterias, éstas hacían los mismo incluso comparado con muestras de camarones silvestre (datos no publicados).

Camarones (*P. vannamei*) alimentados con diferentes fuentes de lípidos (soya, carne, aceite de linaza, pescado y una combinación de éstos) no mostraron diferencias significativas en la diversidad alfa ni entre los principales grupos de bacterias detectados (Zhang *et al.* 2014) aunque si en el crecimiento de los camarones. Una vez mas, la alta variabilidad entre los organismos, enmascara cambios significativos que pudiera haber.

Pre y probióticos

Una práctica común es el usar microorganismos benéficos (probióticos) o nutrientes que promuevan el desarrollo de estos microorganismos benéficos (prebióticos) en el cultivo del camarón, sin embargo, son también pocos los estudios metagenómicos que analicen el efecto de estos aditivos en la comunidad intestinal del camarón. A pesar que los Firmicutes no son los principales miembros de la comunidad intestinal y que comúnmente tampoco existen en organismos silvestres, miembros de este grupo son los que se han usado como probióticos, quizá por que se extrapolo la idea de organismos terrestres, principalmente mamíferos. Dietas fueron suplementadas con dos cepas de bacterias lácticas, *Lactobacillus pentosus* y *Enterococcus faecium* así como sobrenadantes de éstas, en ninguno de los tratamientos se observaron diferencias significativas en el número de phyla observados, excepto en las actinobacterias en solo unos de los tratamientos (Wang, 2016). Mas importante, no pudieron detectar secuencias de las especies bacterianas añadidas en ninguno de los tratamientos, quizá por la escasa o nula capacidad de este tipo de bacterias de colonizar o permanecer en el contenido intestinal, pero no se analizó si existían en la mucosa o epitelio intestinal. Sin

embargo, es sistemas de cultivo en los que se usa como probióticos una mezcla de gamaproteobacterias, bacilliales (Firmicutes) inoculados directamente en el estanque de cultivo, se obtuvo mayor riqueza en los intestino de camarones colectados en aquellos estanques con el probiótico que en los que no lo recibieron así como poblaciones significativamente diferentes de bacterias, favoreciendo las proteobacterias y disminuyendo los Firmicutes (Vargas-Albores *et al.* 2017).

Conclusiones

Hay fuertes evidencias que los organismos marinos, principalmente peces y camarones, tienen unos grupos de bacterias que están presentes en sus tractos digestivos, lo que se conoce como microbiota núcleo y esta microbiota no se modifica mucho con cambios en su entorno, incluidas diferentes dietas. Esta microbiota es seleccionada por el ambiente intestinal. Sin embargo, también existe una microbiota que podemos llamar accesoria que si puede varía significativamente como respuesta a cambios en el entorno intestinal, como son el uso de diferentes dietas.

Es importante considerar la gran variabilidad que existe en la microbiota intestinal entre organismos al diseñar experimentos en los que se quiere determinar el efecto de un parámetro. Es recomendable tener el mayor número de réplicas posible e incluso darles seguimiento individual a los organismos para registrar el cambio de la microbiota o microbioma en el individuo y no necesariamente en la población (tanque).

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