UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

FACULTAD DE CIENCIAS BIOLÓGICAS

THESIS

EFFECT OF THE COMMERCIAL CITRUS BASED AND CHEMICAL PRESERVATIVES IN COMBINATION AGAINST THE GROWTH OF *Campylobacter jejuni in vitro* AND IN A FOOD MODEL

FOR

LAIJU KUZHIPPILLYMYAL PRABHAKARANKUTTY

AS A PARTIAL REQUIREMENT TO OBTAIN THE MASTER DEGREE IN SCIENCE WITH MICROBIOLOGY IN ORIENTATION

APRIL 2016

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This work was realized in the Laboratory of Biochemistry and Genetics of the Microorganisms of the Department of Microbiology and Immunology of the Faculty of Biological Sciences under the direction of Dra. Luisa Yolanda Solís Soto and in coordination with Dra. Norma L. Heredia Rojas and Dr. José Santos García and the support of Consejo Nacional de Ciencia y Tecnología (CONACYT)

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CONTENTS

Section **Page**

List of the Tables

List of Figures

Symbols and Abbreviations

FDA	Food and Drug Administration
USDA	United States Department of Agriculture
USA	United States of America
US	United States
STEC	Shiga-Toxin Producing *E. coli*
CDC	Centers for Disease Control and Prevention
NaCl	Sodium chloride
VBNC	Viable but Non-Culturable Condition
MBC	Minimal Bactericidal Concentration
CFU	Colony Forming Units
TSP	Trisodium Phosphate
FIC	Fractional Inhibitory Concentration
CO_2	Carbon dioxide
µl	Microliter
ml	Milliliter
g	Gram
°C	Celsius degree
%	Percentage
±	More or less
≤	Less than or equal to
≥	Greater than or equal to
Σ	Sum
2X	2 times

Abstract

Campylobacter is common Gram-negative bacteria associated to foodborne bacterial gastroenteritis in humans and currently is the number 3rd due to *Salmonella* and *Norovirus* are more common.

Methods to control *Campylobacter* contamination in foods are not completely successful. The poultry field plays an important role, since chicken are the most common host of these bacteria due to their high body temperature and there is an increase in the consumption of poultry meat worldwide. Plant extracts, essential oil and volatile products originated from plants secondary metabolism have a wide application as food preservatives and flavorings as well. In our study, we used three commercial citrus-based products that are already in use separately, combine and evaluated at different concentration the antimicrobial activity of them against two different *Campylobacter* strains. Finally, we evaluated the effect of the commercial preservatives against any organoleptic characters of the marinated chicken wings using a sensory analysis. The MBC of TSP was 0.5± 0.04%, Citrosan 0.05± 0.0006% and Citrol-K-Ultra® 0.0006± 0.0001% against *C. jejuni* in vitro. The MBC obtained of the combinations of TSP-Citrosan was 0.4%- 0.03%, TSP-Citrol-K-Ultra® 0.3% - 0.0003% and Citrosan-Citrol-K-Ultra® 0.4% - 0.0005%. In the chicken meat, the combinations 2%TSP, 0.3% Citrosan and 0.05% Citrol-K-Ultra® and the combinations of 0.3% Citrosan and 0.05% Citrol-K-Ultra® showed a complete reduction of *C. jejuni* by 48 hours. The sensory analysis showed no significant difference among the different combinations when compared with the chicken without any preservatives.

Introduction

The microbial safety of food continues to be a major concern to consumers, regulatory agencies, and food industries throughout the world. Because the microorganisms are always associated with harvested plants and slaughtered animals, the basic raw materials of the food industry. Except for the foods that are heat processed to the degree that makes them sterile (ultra-pasteurized milk), microorganisms are usually associated with all the food products. Although a few microorganisms can bring about desirable changes in food, others spoil the foods by deteriorating their organoleptic quality or by production of toxins or any secondary metabolite.

Species within the genus *Campylobacter* have emerged over last decades as significant clinical pathogens of human public health concern. This microorganism is responsible for 400-500 million cases of infection each year worldwide, in which approximately 95% are cause by *C. jejuni*, or *C. coli*. Campylobacteriosis, the acute gastrointestinal illness caused by several *Campylobacter* species has been describe as an emerging foodborne disease, which presents symptoms such as acute diarrhea, abdominal pain, and headache. The poultry meat is the main vehicle for human Campylobacteriosis (Heredia *et al.,* 2009).

Many food preservation strategies such as chilling, freezing, water activity reduction, nutrient restriction, acidification, fermentation, pasteurization or synthetic antimicrobial have been used traditionally for the control of microbial spoilage in foods but the contamination of food and their spoilage due to microorganisms is a problem that is yet to be controlled adequately. The safety and shelf life of food ingredients can also be improve by application of novel technologies like packaging in controlled or modified atmosphere, activated films, no-thermal treatments, irradiation etc., to avoid or delay microbial growth. However, most of these procedures may cause loss of organoleptic properties of foods and in consequence to reduce consumer acceptability. Therefore, the consumer demands are increasingly focusing on minimally processed food products, with less use of synthetic preservatives and at the same time without compromising food safety. Although synthetic antimicrobials are approving in many countries, the recent trend has been for use of natural preservatives due to the adverse health effect of synthetic ones. Therefore, alternative sources of safe, effective and acceptable natural preservatives need to be explored (Singh *et al.,* 2012).

Antecedents

Campylobacter and Campylobacteriosis

Campylobacter species are important bacterial pathogenic agents that can cause human gastroenteritis and are transmitting mostly through foods of animal origin (Zoonotic) (Labbe *et al.,* 2001). *Campylobacter* was initially classified as *Vibrio* species due to its spiral morphologies, and later, Sebald and Veron (1963) postulated the new genus *Campylobacter*. The family *Campylobacteriaceae* consists of the genus *Campylobacter*, *Arcobacter*, and *Bacteroides ureolyticus* and it occurs primarily as commensals in humans and domestic animals (Snelling *et al.,* 2005). There are 17 species serotypes within the genus *Campylobacter*, which can be divide into more than 600 Penner or serotypes (according to its heat stable antigen) and more than 100 Lior serotypes (according to its heat labile antigen). All clinically relevant *Campylobacter* species are considering thermotolerant (can growth at 42°C) in nature. The thermophilic species include *C. jejuni, C. coli, C. lari, C. upsaliensis* and *C. fetus*, while the non-thermophilic species include *C. concisus, C.curvus, C. gracilis, C.helveticus, C.hominis, C.hyointestinalis, C.showae, C.sputorum* and *C. rectus* (Heredia *et al.,* 2009).

Campylobacter is a fragile organism in the environment and requires special growth conditions. It grown *in vitro* with partial oxygen tension (2-10%); however, it exhibits great flexibility in its adaptation mechanisms to survive environmental stresses, such as temperature shift, oxygen tension and nutrient depletion, which usually could occur during transmission between the environment and animals hosts and within the host's intestine. This adaptability is attributable to the genetically, metabolically and phenotypically diverse population structure of *Campylobacter* and its capability to adapt in response to challenges. Even more, evidences indicate that *Campylobacter* strains could present phenotypic and physiological differences between strains grown under the same conditions (Heredia *et al.,* 2009).

Members of the family *Campylobacteriaceae* are typically motile with a characteristic *corkscrew*-like motion via a single polar unsheathed flagellum at one or both ends of their cells.

This species require complex growth media as it is not able to oxidize or ferment carbohydrates and has no lipase or lecithinase activity. *Campylobacteraceae* obtain energy from amino acids, or tricarboxylic acid cycle intermediates, oxidase activity is present in all *Campylobacter* species except *C. gracilis*. Members of these genera have small genomes (1.6-2.0 megabases) and can establish long-term associations with their hosts, sometimes with pathogenic consequences (Butzler, 2004; Snelling *et al.,* 2005).

Thermophilic *Campylobacter* species are able to grow between 37 and 42°C, but incapable of growth below 30°C (absence of cold shock protein genes which play a role in low-temperature adaptation), with an optimum temperature of 41.5°C. Levin suggested that these organisms should be referring as "thermotolerant" since they do not exhibit true thermophilia (growth at 55°C or above). However, according to De Cesare *et al.,* (2003), *C. jejuni* survived more than 4h at 27°C and 60-62% relative humidity on some common clean or soiled food contact surfaces. These characteristics reduce the ability of campylobacters to multiply (i) outside of an animal host and (ii) in food during their processing and storage. Growth does not occur in environments with water activity (a_w) lower than 0.987 (sensitive to concentrations of sodium chloride greater than 2% w/v), while optimal growth occurs at a_w= 0.997 (approximately 0.5% w/v NaCl) (De Cesare *et al.,* 2003).

Thermotolerant *Campylobacter*, *C. jejuni/coli*, constitutes the most frequent cause of intestinal infections worldwide, causing Campylobacteriosis (ECDC, 2013). The infective dose for Campylobacteriosis is considerably low, only approximately 500 cells. Following ingestion, motile bacteria reach the mucus layer in the gut. *C. jejuni* passes through the duodenum and exposed to bile secretion. Bile resistance primarily mediated by the CmeABC multidrug efflux pump. Chemotaxis and a requirement for iron drive the bacteria to reach to the epithelial surfaces where they colonize. Toxin production causes cell damage, inflammation, and fluid loss resulting in diarrhea, which appears in 2 to 5 d (Ray and Bhunia, 2013). Adherence may be required for this bacterium to resist intestinal peristalsis and expulsion. To date flagella and motility are the most well defined colonization factors. Flagella allow the bacteria to penetrate the mucous layer covering intestinal cells using their polar flagella and corkscrew motion (Heredia *et al.,* 2009). The main symptom observed is diarrhea, which can vary from limited to voluminous stools that

may be watery or bloody. Another frequent digestive tract symptom is abdominal pain, whereas vomiting is uncommon. Fever, headache, asthenia, and anorexia are also present and may precede diarrhea. *Campylobacter* are enteroinvasive bacteria, which lead to colitis and, in some instances, resemble inflammatory bowel disease, when pain is major feature, of the infection, differentiation from appendicitis may be difficult. Normally the disease will develops two to three days after ingestion of contaminated food and the symptoms resolve themselves within a week (ECDC, 2013).

However, occasionally infection leads to death in infants and young adults (5% of estimated food-related deaths). Infections occur at all ages, although peaks are described for children below the age of four and people between 15 and 39 years. This bacterium has been identifying as an important risk factor for the development of inflammatory bowel disease. Local complications also has been associated with this bacterium such as cholecystitis, pancreatitis, and peritonitis rarely occur, and the immunoproliferative small intestine disease. Bacteremia is detecting in less than 1% of patients and it occurs in most often in patients whose immune system is severely compromising. Some patients develop erythema nodosum or polyarthralgia (reactive arthritis). Extra-intestinal infections including, meningitis, osteomyelitis, and neonatal sepsis are rare. *Campylobacter* infections are also associating with post-infectious complications, including Reiter syndrome and Guillain-Barre syndrome (which is an acute polyneuropathy that affects the peripheral nervous system, where the most typical symptom is an ascending paralysis beginning in the feet and hands and migrating towards the trunk and in some cases a change in sensation or pain as well as dysfunction of the autonomic nervous system is observed) (Heredia *et al.,* 2009).

Due to the absence of a suitable animal model, the virulence properties of these bacteria have mostly investigated using *in vitro* models. This in part has limited our knowledge of the pathogenicity of this organism (Wassenaar, 2011). The main route of *C. jejuni/coli* human infections is through improperly handled or undercooked poultry where are illness caused by *C. lari* and *C. upsaliensis* may be due to proximity to water and shellfish and handling of pets, livestock or livestock carcasses (Garcia and Heredia*,* 2013).

Treatments with antibiotics such as macrolides and fluoroquinolones are usually administering only in severe infections in infants, the elderly immunocompromised individuals and patients with extra intestinal manifestations (Garcia and Heredia, 2013).

Poultry Industry

Consumption of poultry meat has increased remarkably over the past two decades due to the perception that it is a "healthier" alternative to red meats (Synder, 1998). Chicken meat comprises a substantial source of a high quality protein in most countries. Chicken meat is rich in essential amino acids along with vitamins and minerals. Lean chicken contains more protein than the same amount of lean roasted beef and the prices of chicken meat are lower than of beef or pork. Additionally, chicken by-products are consuming widely due to their low price, special taste, and the short time requirement for preparation (Silvan *et al.,* 2011).

However, an increase in the consumption of poultry products has accompanied by an increase in food-borne illnesses associated with poultry. Chicken and other types of poultry have higher pathogenic and spoilage bacterial counts than most other foods. Pathogenic bacteria associated with poultry include *Salmonella* spp., *C. jejuni, Staphylococcus aureus* and *Listeria monocytogenes.* In fact, poultry represents the most important food source of *Salmonella* and *C. jejuni* (Sallam and Samegima, 2004).

The avian species are the most common hosts for *Campylobacter* spp. probably because of their high body temperature. Although, all commercial poultry species can carry *Campylobacter* spp., the risk is greater from chicken, because of the large quantities consumed (Silvan *et al.,* 2011). The intestinal tract of chicken, especially the cecum and colon, can harbor a large number of *Campylobacter*. During processing, the intestinal tract may leak or rupture and the contents transferred to the carcass (Berrang *et al.,* 2001). The highest prevalence of *Campylobacter* in poultry flocks at the pre-harvest level is further exacerbated due to multiple opportunities for cross-contamination to occur during slaughter and processing. The high throughputs of modern poultry slaughter plants have necessitated the development of automated equipment in the stages

of scalding, plucking and evisceration for example. The net effect of processing large numbers of carcasses from different sources very often leads to the dissemination of enteric pathogens including *Campylobacter* from the early stages of the slaughtering process. Also, as skin in normally not removed from dressed carcasses, large numbers of *Campylobacter* cells can remain in situ on the finished raw product thus increasing the likelihood of exposure to the consumer (Moore *et al.,* 2005).

Wills and Murray (1997) realized a study to investigate the effect of environmental temperature over different seasons on the survival of *C. jejuni* in poultry. They demonstrated that *Campylobacter* spp. was present concern in poultry especially during the warmer month (May - October). During these months, 87-97% of the samples tested were positive for *C. jejuni*. The lowest numbers of positive samples were obtaining in December and January (7 and 33% respectively). It was also reporting that there is substantial variability in the intestinal colonization of *C. jejuni* across different broiler flocks at different ages in the production cycle (Moore *et al.,* 2005).

Control strategies

As we mentioned before, *C. jejuni* is a fastidious pathogen that can only grow at 30-45°C in a microaerophilic atmosphere, the pathogen also lacks many stress-responses mechanisms commonly found in other Gram-negative bacteria. Despite this sensitivity to stresses found outside the host, *C. jejuni* is prevalent in poultry houses and slaughter facilities. Different hypothesis have been put forth to explain this case including the suggestion that *C. jejuni* survives in the environment by forming biofilms and/or entering to the viable-but non-culturable (VBNC) state (Magajna and Schraft, 2015).

C. jejuni can form monoculture biofilms or establish in pre-existing biofilms of strong biofilm producers, such as *Pseudomonas* spp., *Flavobacterium* spp., *Corynebacterium* spp., *Staphylococcus* spp., or *Enterococcus* spp. Such biofilms can develop it in food processing environments, in drinking water systems and/or in water systems of poultry houses. *C. jejuni* cells in biofilms are very resistant to environmental stresses and to many disinfectants and they

can survive aerobic and low-temperature stress twice more than the planktonic cells. *C. jejuni* can detach it from biofilm in a food production environment, this lead to contamination of product in water distribution systems; detached biofilm clusters may cause infection of humans or colonization of poultry (Magajna and Schraft, 2015). In addition, actually is now recognize that campylobacters can attain the state of Viable but Non-cultivable state (VBNC), that can lead to under-estimation or no-detection of the organism by culture-based techniques, yet cells in this state can still infect susceptible hosts. However, campylobacters are sensitive to drying or even low humidity's, freezing and freeze-thaw stress, oxygen, etc., therefore the control techniques should take into account this characteristic. Since poultry, especially of chicken as a widely consumed and relatively cheap source of meat, is the mainly source of human Campylobacteriosis, this is the focus of efforts to reduce human disease (Silvan *et al.,* 2011).

a) Sodium Hypochlorite

It has used in poultry processing for more than 40 years to reduce the bacterias that may deteriorate them, controlling the spread of pathogens and prevent the buildup of microorganisms on equipment such as scalding tanks. However, water chlorination is not effectively to reduce bacterias attached to chicken skin (Keener *et al.*, 2004). In 8 hour in chilled water with 10 ppm of chlorine, the reduction of *C. jejuni* and *Salmonella* Typhimurium was <0.5 log CFU/ml while than using 50ppm of chlorine was 4-5.5 logs CFU/ml reduction (Yang *et al.,* 2001).

b) Trisodium phosphate (TSP)

Phosphate have been using as antimicrobial surface treatment agent to decrease populations of pathogens, prevent growth of spoilage microorganisms, and extend the shelf life of fresh poultry. In particular, Trisodium Phosphate (TSP) treatment yields superior antimicrobial effect compared to other phosphates (Sallam *et al.,* 2004). The use of TSP is to eliminate the need of off-line reprocessing. This compound is white, free-flow crystalline that complies with the specifications of the Food Chemical Codex (Keener *et al.,* 2004). TSP is a generally recognized as safe substance by the US Food and Drug Administration and has approved by the US Department of Agriculture – Food Safety and Inspection Service (USDA-FSIS) at levels of 8-12% as an antimicrobial agent on raw chilled poultry carcasses that have

been passed for wholesomeness. Treatment of poultry carcasses with TSP was effective in reducing populations of foodborne pathogens including *Salmonella*, *Campylobacter*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* as well as spoilage bacteria including *Pseudomonas* and *Lactobacillus* (Sallam *et al.,* 2004). The mechanism of action is proposing on its high alkalinity in solution (pH 12.1) that can disrupt cell membranes and remove fat films causing the cell to leak intracellular fluid. It can also act as a surfactant contributing to elimination of bacteria not yet strongly adhered to the surface of poultry skin (The EFSA Journal, 2005). Several reports have mentioned that is possible to use TSP at different concentration as a dipping by immersion or as spraying method. Although the concentration normally used in the poultry industry is between 8-12%, the lowest effective concentration for microbial control is 8% (The EFSA Journal, 2005). However, a major concern is that exposure to sublethal concentrations of TSP may increase bacterial tolerance to food processing interventions, preservation treatments and antibacterial conditions within the human hosts (Riedel *et al.,* 2012).

c) Sodium chloride (NaCl)

Sodium chloride is a naturally occurring mineral, acts as preservative and flavor enhancer. The use of NaCl in meat to increase shelf life and enhance flavor is an old practice. Addition of NaCl to meat has been associated with antimicrobial properties and at the same time, it also used to improve water-holding capacity and results in subsequent improvements in purge loss and cooking yield (Sallam *et al.,* 2004). Has reported that 0.5% NaCl shows a reduced growth or increased rated of death of the *C. jejuni* (Doyle *et al.,* 1981).

d) Chlorine dioxide (ClO_2)

Chlorine dioxide is an antimicrobial compound recognized for its disinfectant properties since the early 1900s. It is a synthetic yellowish green gas with chlorine-like odor. It functions independent of pH and provide excellent control at a fraction of the chlorine dosage because it can use at much lower doses. The smaller dosage also makes chlorine dioxide more cost-effective. Chlorine dioxide kills microorganisms by disrupting transport of nutrients across the cell wall. The additive may use to control the microbial population in poultry processing chill water in an amount not to exceed 3-ppm residual chlorine dioxide (Keener *et al.,* 2004).

e) Acidified Sodium Chlorite

Sodium chlorite, at a concentration of 500-1200 mg/L activated with any acid approved for use in foods at levels sufficient to provide solutions with pH values in the range 2.3-2.9 for either a 15s or 5-8s in spraying or dipping respectively. In the case of immersion in chilling water, the concentration is up to 150 mg/L at pH between 2.8-3.2 (The EFSA Journal, 2005).

f) Organic acids

Several organic acids have proven effective in poultry processing such as Acetic, Lactic, Citric and Succinic acid. Okrend and others (1986) added 0.1% acetic acid to scald water and observed a reduction of populations of *Salmonella* Typhimurium and *C. jejuni* from 0.5 to 1.5 $\log_{10}$ CFU/ml. Bautista and others (1995) studied the effect of lactic acid, chlorine (50 ppm), and TSP sprays under various pressures on treating turkey carcasses. They observed that 1.25% and 4.25% lactic acid caused a 2.4 and 4.4 $\log_{10}$ reduction in aerobic plate count (Keener *et al.,* 2004).

g) Irradiation

The biological effect of ionizing radiation on cells can attribute to direct interactions with critical cell components and to indirect actions by molecular entities such as free radicals formed in the water. The DNA of the cell is the most critical target of ionizing radiation, and the inactivation of microorganisms is primarily due to damage to the DNA. The FDA and USDA have approved irradiation of chicken at a maximum dose of 3kGy to control foodborne pathogens such as *Salmonella* and *Campylobacter* (Keener *et al.,* 2004).

h) Others

Despite to the above, has been reported many studies about the antimicrobial activities of different components against *Campylobacter*. For example, in our lab we have done different studies with the citrus-based compounds. Castillo in , 2014 did the comparative studies using Citrol- K Ultra, which showed better antimicrobial activity against *C. jejuni* compared to the natural antimicrobials used in another study by Valtierra and group in our lab itself.

Koolman *et al.,* 2014 evaluated the efficacy of 12%(w/v) TSP, 2% (w/v) citric acid (CA) and 5% Capric acid salt (CP) in reducing *Campylobacter.* These chemicals are also using in different combinations (TSP+CA, TSP+CP and CA+CP) to determine if sequential treatments would enhance microbial reduction. In this case, TSP and CP gave largest *C. jejuni* reduction (1.9-2.3$\log_{10}$ and 2.2-2.4 $\log_{10}$ CFU/cm^2 respectively).

Antimicrobials in food

In a study done by Capita *et al*., 2002, use Trisodium phosphate to reduce levels of bacterias in poultry. In that study, using TSP was capable to obtain a microbial reduction on poultry against *Salmonella, Coliforms/Escherichia coli, Enterobacteriaceae*, *Campylobacter, Pseudomonas*, total count, *Listeria, Staphylococcus aureus* and *Lactobacillus*.

Valtierra *et al*, in 2009 studied the antimicrobial activity of extracts from 28 edible plants against *C. jejuni* and *C. coli in vitro* and in a poultry skin model. In their study, the mixtures of the lime, plum and sour orange peel extracts showed a significant reduction of the bacterial count in chicken wings. In the next year, Pisernik *et al* 2010 tested the antimicrobial effect of rosemary extracts and the bacteriocins nisin against *C. jejuni* at a low storage temperature without short-term pre freezing. The results in chicken meat showed a synergistic effect of freezing effect and plant extract antimicrobial activity, which showed a reduction of the cell number by more than 2.0 logs.

In the year 2013, Garcia-Heredia *et al*, worked on the efficacy of citrus-based disinfectants to control growth, biofilm formation and *swarming* of *Salmonella* and the efficacy of Citrik Agro® to disinfect contaminated parsley. Citrik Agro® showed more than two reduction log of *Salmonella* in parsley. Koolman *et al*, 2014, evaluated the efficacy of 12% Trisodium phosphate 2% Citric acid and 5% Capric acid sodium salt in reducing *Campylobacter*, total viable and total *Enterobacteriaceae* counts on poultry. These chemicals also used in various combinations.TSP+ CP was the most effective combination treatment 2.9-log10 cfu cm^2 for reducing *C. jejuni*.

However very little have done by combining these agents. In this study, we are going to focus on the effect of using a combination of the different chemical agents that have been using in the industry already as antimicrobial agents. By combining, these preservatives may help us to reduce the amount of each preservative that have to add to the poultry meat.

Definition of the problem and justification

Consumption of chicken meat generally accepted as dominant risk factors for sporadic *Campylobacter* species infections among humans (Ingrid H. M *et al.,* 2012). The trend in *Campylobacter* reported rates is stable; most cases are sporadic, although small outbreaks are reported and poultry meat was the most frequently identified outbreak vehicle in reported outbreaks in 2011 (www.ecdc.europa.eu.com).

The true incidence of gastroenteritis due to *Campylobacter* species is underestimated and several approaches are using to try to estimate it. In different studies done in United Kingdom and Netherlands, the incidence of Campylobacteriosis was estimate to be 9.3 per 1000 hab/years (for 2008-2009) and 5.8 per 1000 hab/years in Netherlands (2009). In USA, it estimated that one out of 30.3 cases reported by FoodNet sites, and that national incidence was 1.3 million cases in 2006 or 4.4 per 1000 (Global view 2012). In 2012, FoodNet identified 19,531 laboratory-confirmed cases of infection, 4563 hospitalization and 68 deaths among 48 million residents of 10 states (15% of the US population), which confirms that Campylobacteriosis is the second most frequent bacterial infection. Comparing this with the incidence rates in 2006-2008 shows, that incidence of *Campylobacter* infection was 14% higher.

So Healthy People 2020 national targets for reducing the rates of infection caused by *Campylobacter, Listeria, Salmonella, Shiga-toxin* producing *E. coli* (STEC), *Vibrio* and *Yersinia* (CDC 2012). According to the Foodborne Disease Active Surveillance Network (FoodNet) USA, in the year 2014 showed a 13.45% of incidence of culture-confirmed cases of *Campylobacter* found (CDC, 2014). The prevalence of *Campylobacter* spp in the fresh chicken at retail in the UK found out by a study (Jorgensen *et al.,* 2015) was 73.3%. A significant proportion (19.4%) of samples had >1000 cfu/g of chicken skin, and this ranged between retailers from 12.9 to 29.9 %.(Jorgensen *et al.* 2015)

The avian species are the most common host for *Campylobacter*, probably because of their higher body temperature. There has been an increase in per capita consumption of poultry (Kenner *et al.,* 2004). During the period 1980 to 2012, per capita poultry meat consumption increased from 26.4 to 54.1 pounds per year (*in senso stricto*, the amount of poultry meat available for human consumption in the US). The increased consumption of poultry products may increase consumer's risks of acquiring foodborne illnesses. Treatment of processed poultry products with antimicrobials is one of the most effective strategies for minimizing consumer's risks associated with consuming poultry products (Shilpa *et al.,* 2015).

Most food products require protection against microbial spoilage during storage. Consumers demand safe natural products and this drives the search of food authorities and researchers for mild preservation techniques to improve microbial quality and safety without causing nutritional and/or organoleptic losses. In this context, natural compounds are gaining a great interest from research and industry, due to the potential to provide quality and safety benefits, with a reduced impact on human health. In addition, utilization of natural active agents promotes the accepted criteria of food sustainability. The numerous experimental applications of essential oils (basil, thyme, oregano, cinnamon, clove and rosemary), enzymes (lysozyme, lactoferrin), bacteriocins (nisin, natamycin), organic acids and salts (sorbic, propionic, citric acid, triphosphate sodium salt) chitosans, to several fresh perishable foods demonstrate that they are well suited to be utilized as preservatives in foods and could be often valid alternatives to synthetic food additives.

Inorganic compounds such as Trisodium Phosphate treatment is officially accepted and widely implemented in the poultry slaughter process and it does not cause undesirable sensory effects detectable by the consumer. In addition, the use of combinations of different food preservation systems such as organic, inorganic and proper temperature could represent a great solution to effective preservatives in poultry industry.

Hypothesis

The commercial citrus-based and synthetic preservatives in combination can inhibit the growth of *Campylobacter jejuni* without affecting the organoleptic properties of chicken meat.

General Objective

To determine the effect of the combination of commercial citrus-based and synthetic preservatives against the growth of *C. jejuni*.

Particular objectives

1. To determine the Minimal Bactericidal Concentration (MBC) of the commercial citrus-based and synthetic preservatives against the growth of the two strains of *C. jejuni*.

2. To determine the effects of the combination of these preservatives against the growth of *C. jejuni in vitro*.

3. To determine and apply it the best combination in a food model and through a sensory evaluation, analyze the changes in the organoleptic properties of the chicken meat.

Methods

Bacterial Strains and the culture conditions

In this study we used *Campylobacter jejuni* NCTC 11168 acquired commercially and *C. jejuni* NADC 5653 kindly donated by Dr. Irene Wesley as controls. These bacterial strains were stored as stock culture at -80°C in crioviales with sterile glycerol (2% v/v).

The strains were activated using an aliquot (50 µl) of the stock culture and inoculated it into 5 ml of the Brain Heart Infusion (BHI, DIFCO), supplemented with Yeast Extract (0.6% DIFCO). These cultures were incubated at 42°C under microaerophilic conditions (10% CO_2) for 48 h and finally an aliquot from these culture was streaked onto Mueller Hinton (MH, DIFCO) agar supplemented with lysate blood (5% v/v) plates and incubated at the same conditions above described.

Preservatives Used

We used three different preservatives acquired commercially in this study: 1) Trisodium phosphate (TSP) (Food Proteins Corporation, S.A, De C.V.) 2) Citrosan (Diken international) and 3) Citrol K- Ultra ® (Corpocitrik S.A. de C.V, Mexico).

Determination of the Minimal Bactericidal Concentration (MBC) in *Campylobacter jejuni* NCTC 11168 and NADC 5653

The method to determine the Minimal Bactericidal Concentration (MBC) was as follows. From the *C. jejuni* culture activated onto MH agar, we selected colonies using a sterile cotton swab and then homogenized in sterile saline solution (0.85% w/v) and adjusted to 74±2 Transmittance units (~1.5 x 10^8 UFC/ml) using a spectrophotometer (Sequoia Turner Model 340). From this culture, an aliquot (20 µl) was homogenized with 980µl of the MH broth (2X) and 1 ml of the corresponding preservative (Citrol K Ultra® 0.0006%, Citrosan 0.05% or TSP 0.5%). This combination were incubated under microaerophilic condition (5% CO_2) at 42°C for 24 and 48h, in which an aliquot (100µl) of any treatments was streaked onto MH plus lysate

blood agar plates using a Driglalsky glass rod and incubated again at the same conditions above described. At the final time of incubation, the colonies of *C. jejuni* were count to determine bacterial survivors.

Determination of the Minimal Bactericidal Concentration of the preservative in Combination for *C. jejuni* NCTC 11168 and NADC 5653

This experiment was did using the Checkerboard method reported by Orhan *et al*. (2005), with several modifications. Our preservatives combinations were TSP-Citrosan, TSP- Citrol-K-Ultra® and Citrosan-Citrol at different concentrations (Table 1). In order to find out the best combination of the preservatives, first we prepared a several stock solutions of each preservative in a high concentration (TSP 10 %, Citrosan 1% and Citrol-K-Ultra®0.01%), and then we combined them in MH Broth and *C. jejuni* 1% (~$1.5x10^6$ CFU/ml v/v). These cultures were incubate under microaerophilic condition (5% CO_2) at 42°C for 0, 24 and 48h, in which an aliquot (100 µl) of any treatments was streaked onto MH plus lysate blood agar plates using a Driglalsky glass and then incubated at the same conditions to 24 and 48h in which we determine the rate of growth of *C. jejuni*. Each experiment did in triplicate. We used a *C. jejuni* culture without treatments as control.

Table 1. Concentrations used of each preservative and their combinations

	Preservative 2				
	Concentration	100%	75%	50%	25%
Preservative 1	100%	100-100	100-75	100-50	100-25
	75%	75-100	75-75	75-50	75-25
	50%	50-100	50-75	50-50	50-25
	25%	25-100	25-75	25-50	25-25

Synergy is more likely to be expressed when the ratio of the concentration of each preservative to the MBC of that preservative will be same for all components of the mixture. ΣFIC (Fractional Inhibitory Concentration) was calculating as follows:

$$\Sigma FIC = FIC\ A + FIC\ B$$

Where:

FIC A: MBC of preservative A in combination / MBC of preservative A alone

FIC B: MBC of preservative B in combination / MBC of preservative B alone

The combination is considered synergistic, when the $\Sigma FIC \leq 0.5$, indifferent when the ΣFIC is > 0.5 to < 2, and antagonistic when the ΣFIC is ≥ 2.

Application in the Food Model

The raw chicken skins obtained from a local supermarket in the metropolitan area of Monterrey, Nuevo Leon. In order to prevent the interference of the normal microflora present in the raw chicken skin, we did a decontamination process, in which we removed all the visible fat from the skin, and then cut it into small pieces (2x2 cm, ~1g) and then put them into a sterile bag (1L capacity). After that the chicken skin, washed 10 times with the tap water (1L each) and 3 times with sterile distilled water (1L each). The pieces washed were exposed to UV light (wavelength 254 nm) for 30 min on both sides and then kept frozen at -20°C for 24 h. To make sure these decontaminated pieces are free of *Campylobacter* and Mesophilic bacterias, we did an analysis using Bolton broth and Campy-cefex agar for *Campylobacter* and Aerobic plate count agar to mesophilic bacterias. This is realized as follows. After the 24 h freezing, we took a piece of chicken skin piece, and incubated into 5ml of Bolton broth for 4 h at 37° C in aerobic conditions and 44 hat 42° C under microaerophilia. From that, we took 100µl and plated onto the Campy-Cefex agar, and then incubated under microaerophilic conditions at 42° C. After 48 hours, we observed the colonies. In the case of *Campylobacter* were present we discarded the assay. The aerobic plate count was done as follows: one chicken skin piece was taken after 24 hours of freezing. This was homogenizing in 9ml of Peptone water for 2 minutes. An aliquot (200µl) from this homogenized was mixed with 1.8 ml saline water, and did the decimal serial dilutions, which then plated on the Aerobic Count Agar plates. These plates then incubated at 37°C for 24 hours in aerobic conditions and counted the colonies.

To inoculate the chicken skin, after 24 h of freezing, we tried two different methods. First, the chicken skin (1 piece) was washing with the TSP (2%, 2ml) for 30 s by immersion and then with distilled water (2 ml) for 30s by immersion before the *C. jejuni* inoculation. In the second method, we made the same that in the first; however, the first step was to inoculate *C. jejuni* and then washing steps done.

The clean chicken skin was introduce into a sterile petri dish and the inoculated with a bacterial cocktail (both strains of *C. jejuni*) adjusted to $1.5X10^8$ CFU/ml and maintained for 10 min at room temperature to allow that C. *jejuni* were attached to skin surface. After that, the inoculated chicken skin pieces were submerging in Citrosan, Citrol-K-Ultra®, or their combination in a ratio of 2 ml for each skin piece. These were incubate at 4°C for 0, 24, 48 and 120 h, which we realized the bacterial count taking off a skin piece from each treatment and homogenized it slowly for 2 min with sterile solution saline (9ml). We made the decimal dilutions, plated onto Campy-Cefex (BD) agar plates, and incubated under microaerophilic conditions at conditions above described. The characteristically colonies of *Campylobacter* were counted at 48 h. A positive control (without preservative) also used in each assay.

Sensory Analysis

The sensory analysis realized using a simple range test according the description for hedonic test described by Meilgaard *et al*, (2007). The analysis done with natural chicken wings, acquired in a commercial supermarket in Monterrey, NL. The chicken wings washed five times with tap water (1L) and then one more time with sterile distilled water (1L). After that, the chicken wings drained and then immersed in the selected concentration of each preservative. The combinations used in the sensory analysis were 2:0.3:0.05 % TSP: Citrosan: Citrol-K-Ultra® (treatment 1), 0.3:0.05% Citrosan: Citrol-K-Ultra® (treatment 2) and only water as a control (treatment 3). For this, we added 250ml of each treatment at 25 chicken wings in a sterile bag (1 gallon). The chicken wings in each treatment were mix and refrigerated at 4°C for 48 h, which then baked at 290°C for 60 min in conventional wave.

The sensory analysis of the cooked chicken wings was done by a panel of semi-trained people composed of the students and professors of the Faculty of Biological Science of the Autonomous University of Nuevo Leon (n=37). Utilized the test questionnaire to assess the order of the preference of the chicken wings as shown in the figure No 1 assigning a rank from 1 to 5 in which the number 1 was indicated as less preferred, whereas the number 5 was indicated as

mostly preferred. A glass of water was given to each person between each sample. (Sturles *et al.*, 2004).

Statistical Analysis.

The graphs are done with the program Sigma Plot version 10. All the results obtained analyzed using the Scheffe test in the IBM SPSS Statistics version 22. In the case of sensory analysis, the method utilized was Duo- trio method and the results analyzed through the Chi-square program.

Análisis sensorial

Pruebe las muestras de la Alas de pollo que se presenten a continuación e indica tu nivel de agrado para cada una de las características marcando con el puntaje de una escala de 1-5 que mejor describa el producto.

1. *Me disgusta mucho*
2. *Me disgusta moderadamente*
3. *No me gusta ni me disgusta*
4. *Me gusta moderadamente*
5. *Me gusta mucho*

No de la muestra	Color	Olor	Sabor	Textura	Aceptación general

Fig. 1 Format utilized for the Sensory analysis

RESULTS

Determination of the Minimal Bactericidal Concentration of the Preservatives alone

The Minimal Bactericidal Concentration was determined for the three preservatives alone against both the strains of *C.jejuni* NCTC 11168 and *C. jejuni* NADC 5653. The CMB for TSP to the two strains of bacterias was 0.5%, Citrosan was 0.05% and Citrol-K-Ultra® was 0.0006%, which is shown in the table.

Table 2. The MBC of the three preservatives (TSP, Citrosan and Citrol-K-Ultra® against *C.jejuni* NCTC11168 and NADC 5653).

	Preservatives ± Standard Deviation		
Strain	**TSP %**	**Citrosan %**	**Citrol-K-Ultra® %**
***C. jejuni* NCTC 11168**	0.5 ± 0.04*	0.05 ± 0.0006	0.0006 ± 0.0001
***C. jejuni* NADC 5653**	0.5 ± 0.04	0.05 ± 0.0006	0.0006 ± 0.0001

***Standard Deviation**

Determination of the Minimal Bactericidal Concentration of the Effective Combination of the Preservatives

We realized the combinations: TSP-Citrosan, TSP-Citrol-K-Ultra® and Citrosan-Citrol-K-ultra® in different concentrations to determine the MBC for a cocktail of both strains of *C.jejuni* using the Checkerboard method. Even though some of these combinations showed indifference among them , where as a very few of them has got synergism between them with a range for TSP-Citrosan 1.4 – 1.6%, 0.5-1.4% for TSP-Citrol-K-Ultra® and 0.2 – 1.6% for the combinations Citrosan-Citrol-K-Ultra® (Table 3.).

In this case, we used the combinations 0.4-0.03% TSP-Citrosan, 0.3-0.0003% TSP-Citrol-K-Ultra® and 0.04-0.0005% Citrosan-Citrol-K-Ultra® to apply in the food model *in vitro*

Table 3. Concentrations used for the combinations 1. TSP-Citrosan, 2. TSP-Citrol-K-Ultra®, 3.Citrosan-Citrol-K-Ultra®

Combination	Preservative	Recommended in industry	MBC alone	MBC in combination
1	TSP	12%	0.5%	**0.4%**
	CITROSAN	0.3%	0.05%	**0.03%**
2	TSP	12%	0.5%	**0.3%**
	Citrol-K-Ultra®	0.5%	0.0006%	**0.0003%**
3	CITROSAN	0.3%	0.05%	**0.04%**
	Citrol-K-Ultra®	0.5%	0.0006%	**0.0005%**

We tested the growth of *C.jejuni* (alone and in a cocktail) along 24 hours when we used the combinations of preservatives and incubated at 42°C under microaerophilic conditions. (The detection limit of our assay was 100 cells per ml). In these combinations, TSP-Citrosan, the bacterial count came to a non-detectable level by the end of the 12h for the cocktail When we did the combination of TSP-Citrol-K-Ultra® the cocktail bacterial count has come down to a non-detectable level by 6 hours of incubation. When we realized the combination of Citrosan-Citrol-K-Ultra®, the cocktail bacterial count has come to a non-detectable level by 24 hours of the incubation period.

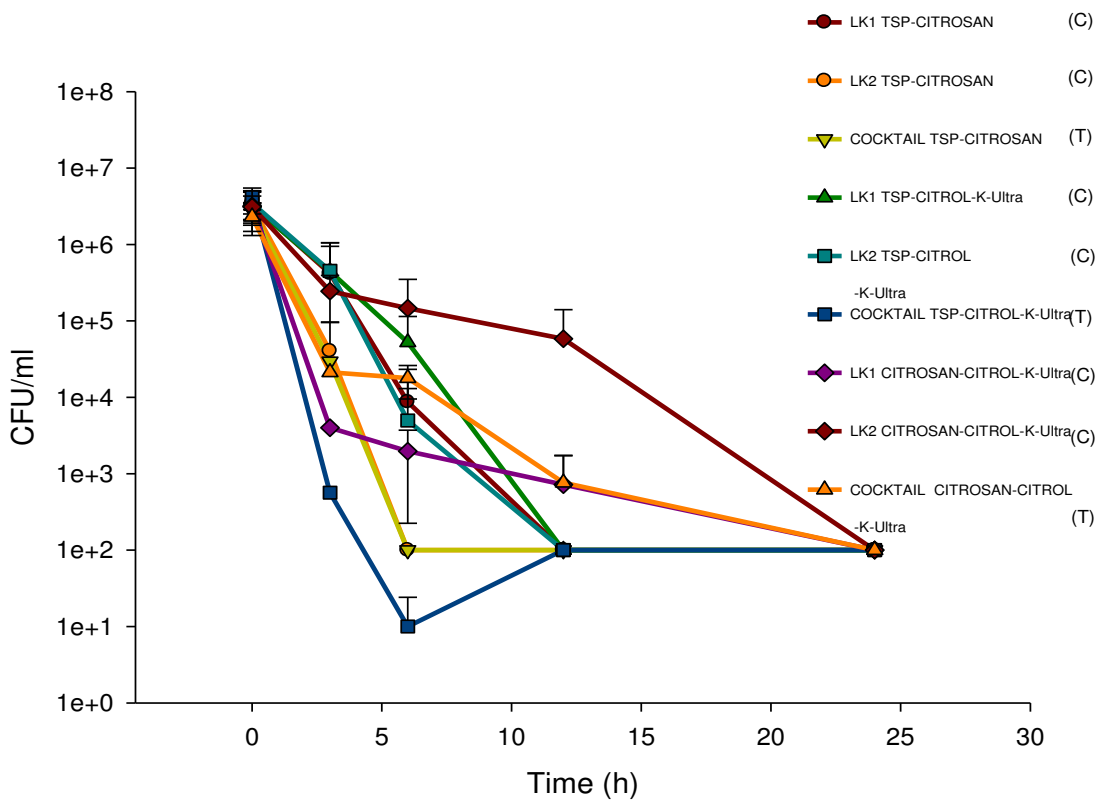

Fig 2. - Combination of the preservatives against the 2 bacterial strains and the cocktail *in vitro.* LK1:*C.jejuni* NCTC11168, LK2: *C.jejuni* NADC5653 (C) Control (T) Test

Calculation of FIC Values

The FIC values calculated according to the procedure, and we got the results in the range of 1.6 to 0.7. Therefore, we can see that all the combinations were indifferent.

Application in the Food Model

According to our results obtained from the different combinations of the preservatives, we decided to use the concentrations 10% above of our MBC to have a good effect of the matrix of chicken skin (as recommended in previous studies). Therefore, we used 4% TSP, 0.3% Citrosan and 0.005% Citrol-K-Ultra®. The first combination was TSP-Citrosan-Citrol-K-Ultra® and the second combination was only with Citrosan-Citrol-K-Ultra®. At the same time, we used other concentration also by reducing TSP and increasing the concentration of Citrol-K-Ultra® and the final concentrations that we tried on chicken skins were TSP 2%, Citrosan 0.3% and Citrol-K-Ultra® 0.05%.

When we analyzed the microbial growth using a combination of the 3 preservatives TSP 2%, Citrosan 0.3% and Citrol-K-Ultra® 0.005% was capable to reduce of 3-4 logarithms within 5 minutes of the application of the mixture of the preservatives, and the bacterial count became less than 100 cells/ml in 24 hours. In the combination Citrosan-Citrol-K-Ultra® (0.3% -0.005%) we obtain a reduction of 1-2 logarithms immediately after the application of the mixture and the bacterial count became < 100 cells/ml in the 48 h.

At the same time we also realized two different methods to the application of TSP according to Keener *et al*, 2004 one mode of action of TSP is that it removes the bacteria that are not yet firmly attached to the skin surface, removal of some surface fat which facilitates the removal of bacteria by the washing process. In the first method, we used the TSP before the inoculation with the bacteria and in the second method; we did the washing of the chicken skins after the inoculation with the bacteria. We could see that the method in which we used the TSP before the inoculation showed much better results in terms of the reduction of the bacterial count in the first hour itself. For example, when we used 2%TSP before the inoculation with the bacteria and then the combination of Citrosan 0.3% and Citrol-K-Ultra® 0.05%, it showed a reduction of the bacterial count by 4 log at the first hour and to a non-detectable level by 48 h of the incubation with the preservatives. Whereas when the TSP 2% used after the inocula applied

and then the combination of Citrosan and Citrol, the bacterias were detectable even after 120 h of incubation (Fig 3 and 4). When we did the combination of only the Citrus based preservatives, we did observed that the bacterial counts were gone down to <100 cells/ml by the end of the 48 h.

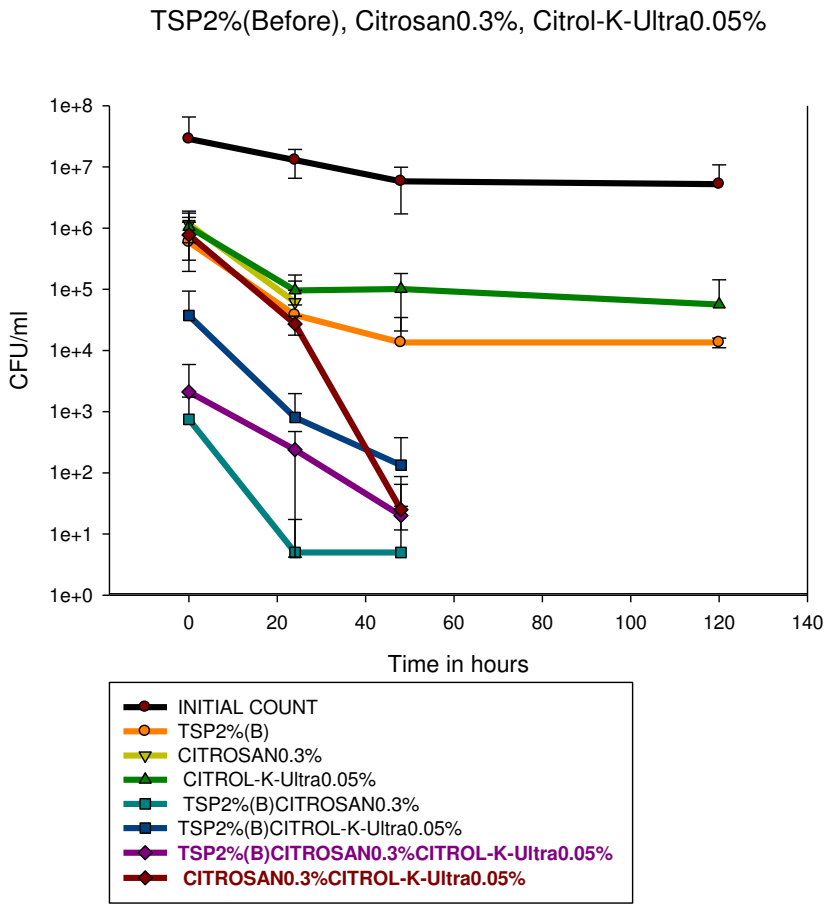

Fig 3. The chicken skin washed with TSP2% before the bacterial inoculation and then the different combinations of Citrosan and Citrol-K-Ultra® were used.(B): Before

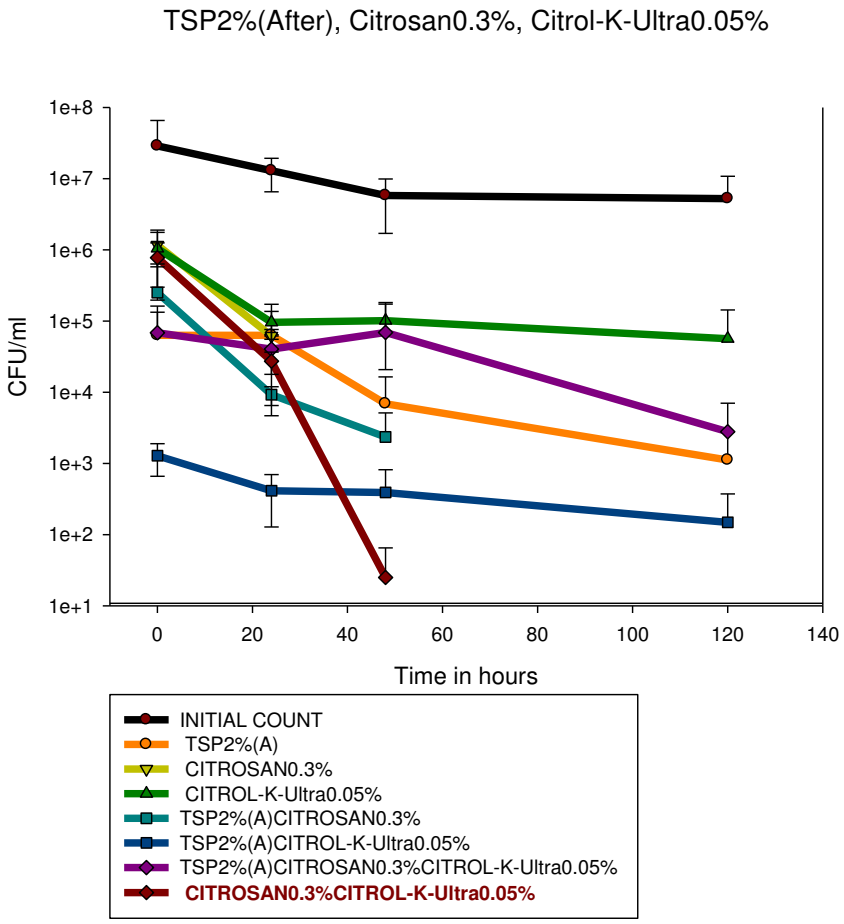

Fig. 4 The chicken skin washed with TSP 2% after the bacterial inoculation and then the different combinations of Citrosan and Citrol-K-Ultra® were used. (A): after

Both the results were checked through the statistical method of Scheffe and there was no significant difference between these two methods ($p>0.05$)

Sensory Analysis

We realized three treatments to the sensory analysis. The treatment one was chicken skin treated with TSP-Citrosan- Citrol-K-Ultra® at the concentrations 2-0.3-0.05% respectively. The treatment two was with Citrosan-Citrol-K-Ultra® at 0.3-0.05% concentrations. A third treatment was a negative control with only water.

The evaluators for the sensory analysis were a semi-trained panel. According to their preference poll, the results showed that with respect to color and texture, the treatment 1 2%TSP, 0.3%Citrosan and 0.05% Citrol-K-Ultra® was more accepted. In terms of odor/flavor, treatment 2 with 0.3% Citrosan and 0.05% Citrol-K-Ultra® was preferable (Fig 5-7).

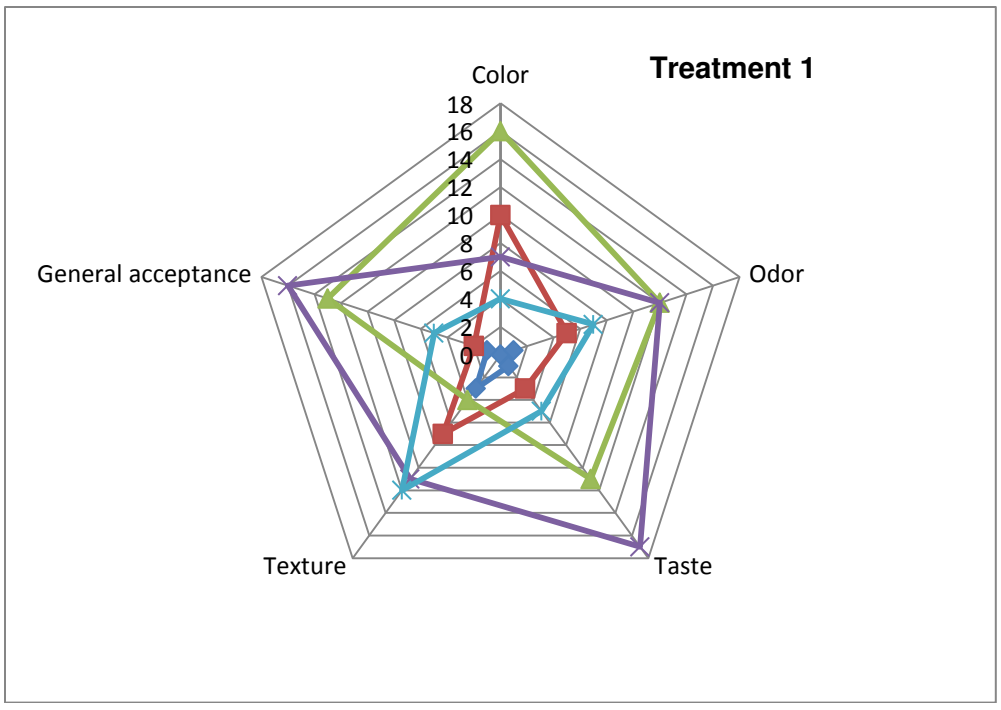

Fig 5 Graphical representation of the 5 variables for the treatment no 1 (2%TSP, 0.3% Citrosan and 0.05% Citrol-K-Ultra®).

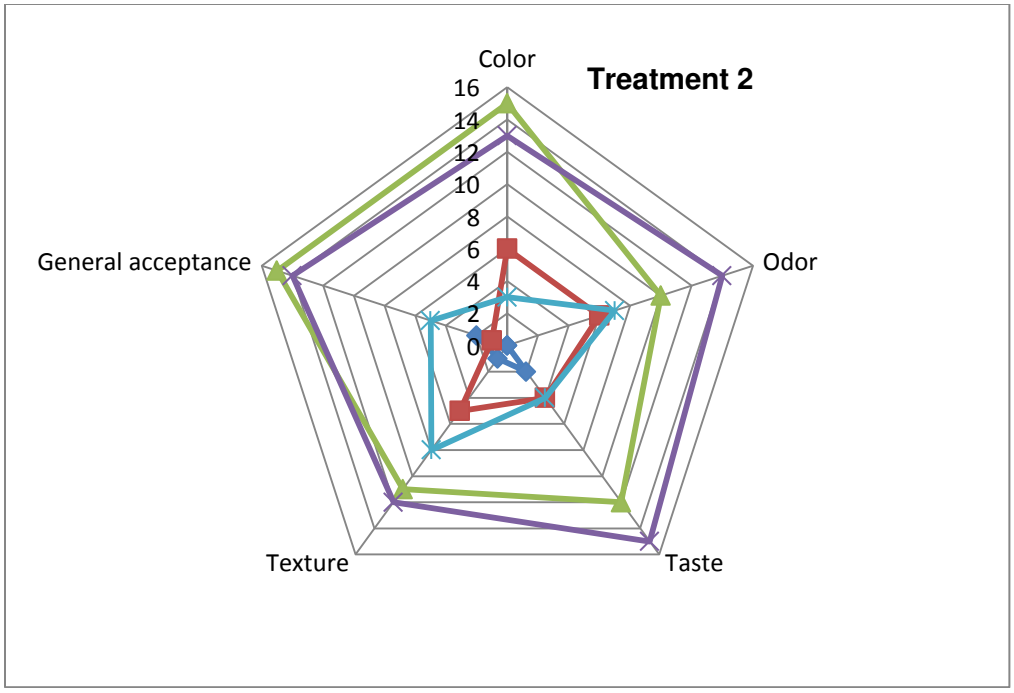

Fig. 6 Graphical representation of the 5 variables for the treatment no 2 (Citrosan 0.3% and Citrol-K-Ultra® 0.05%).

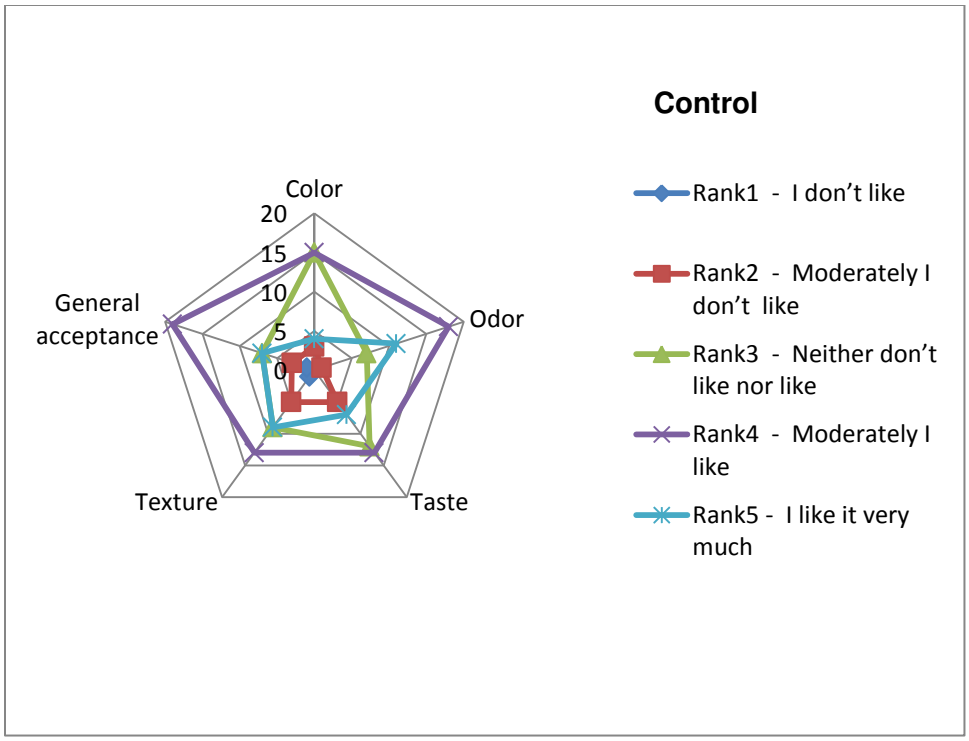

Fig. 7 Graphical representation of the 5 variables for the control (without any treatment)

DISCUSSION

Treatment of processed poultry products with antimicrobials is one of the most effective strategies for minimizing consumer's risk associated with consuming poultry products. The antimicrobial activity of a treatment agent against target microorganisms varies with the concentration of the agent. In many cases, high concentrations are needed to achieve the desired antimicrobial effect. It is well known that the higher concentrations of antimicrobials might adversely affect the product in terms of its sensory attributes (Samant *et al.,* 2015).

Treatment of poultry carcass with TSP was effective in reducing the population of food-borne pathogens including *Salmonella, Campylobacter, Escherichia coli* O157:H7*, Listeria monocytogenes, Staphylococcus aureus* as well as spoilage bacteria including *Pseudomonas* and *Lactobacillu*s (Sallam and Samejima 2004). For over 20 years the efficacy of TSP, at concentrations of 8-12% as a poultry carcass decontaminant has known. The principal mode of decontamination based on physical detachment of bacterial cells and not an antimicrobial effect (H.Meredith *et al.,* 2013).

In our study, the MBC alone obtained for the 3 preservatives that we used in this study are following. 0.5% for TSP, 0.05% for Citrosan and 0.0006% for Citrol-K-ultra®. This could be compare with the industrial usage, that 12% for TSP, 0.3% for Citrosan and 0.5% for Citrol-K-Ultra® respectively over *Campylobacter jejuni*. With these results, we can conclude that even with a very low concentration of the preservatives, we can reach to an effective antimicrobial activity of these preservatives.

Other researchers (Koolman *et al.* 2014) treated chicken legs with TSP 12%, Capric acid 5% or Citric acid 2%, led to reduction of 1.9 $\log_{10}$ CFU/cm^2 with TSP, 1.0 $\log_{10}$CFU/cm^2 with CA and 2.2 $\log_{10}$CFU/cm^2 with CP in *C. jejuni*, 0.9$\log_{10}$ CFU/ cm^2 for TSP, 0.7 $\log_{10}$ CFU/cm^2 for both CA and CP for both Total Enterobacteriaceae counts (TEC) and Total viable counts (TVC). While further reductions were, obtain by treating the drumsticks with combinations of these chemicals, the results were varied and depending on the chemical combinations used. Future work could involve, using different combinations of chemicals to reduce the microbial burden on carcasses or determining the safety of the chemical, the potential risk of by product

formation and if it affect on the organoleptic qualities of the food product. (Koolman *et al.* 2014).

When we combined these preservatives, in different concentrations, we got a MBC lesser than we used them alone. In the combination of TSP-Citrosan, we got an MBC of 0.4% for TSP and 0.03% for Citrosan. When we combined TSP and Citrol-K-Ultra®, we got an MBC of 0.3% for TSP and 0.0003% for Citrol-K-Ultra®, whereas when we did a combination of only Citrosan and Citrol-K-Ultra®, we got an MBC of 0.04% for Citrosan and 0.0005% for Citrol-K-Ultra®.

The FIC values were obtain for the combinations of the preservatives to check if there was synergy, indifference or antagonism among them according the concepts and formulas described by Hall *et al.,* (1983) and along with Orhan *et al.,* (2005). The results showed an average range of 1.6 to 0.7, which corresponds to within the limits of the indifference (< 0.5 - > 2.0). Even though there is no synergy exist among these preservatives, but having an indifferences, shows that these preservatives can be mixed without having any effect between them.

In a research realized by Valtierra *et al* (2009), out of the 28 edible plant extracts they analyzed 9 exhibited antimicrobial activity. From these, the 3 most active extracts were selected to combine them to lower the amounts of each compounds that could be used to inhibit the growth of *Campylobacter* and hence the sensory properties of foods would be less altered. The 3 extracts they selected were lime, plum and sour orange peel extracts, which they mixed in different manners showed a significant reduction of *Campylobacte*r. At 48 h of incubation, the population of *Campylobacter* diminished to an undetectable level (<10CFU/ml)

We utilized chicken skin as the food model, since most of the contamination during the evisceration process will be finding in the skin surfaces. We utilized different concentrations on chicken skin to find out the least concentration of the synthetic preservative and the citric-based preservatives that will be effective against *Campylobacter jejuni*. As we know that, the food matrix is so different from the *in vitro* analysis.

There are several mechanisms for the TSP mode of action: surfactant properties, destructive effect on bacteria at the high pH (pH 11); removal of bacteria that are not yet firmly

attached to the skin surface; removal of some surface fat, which facilitates the removal of bacteria by the washing process; and an effect on the bacterial cell wall (Keener *et al.* 2004,). Therefore, we realized 2 different procedures for the utilization of TSP. In one procedure, we utilized the TSP before the bacterial inoculation on the chicken skin, where as in the other procedure we utilized the TSP after the inoculation. After that, the inoculated chicken skin as exposed with Citrosan and Citrol-K-Ultra® in this case and kept the refrigerator at 4°C to stimulate the normal conditions of the preservation of chicken meat. We also realized a procedure where we omit the use of the TSP and for this; we kept a positive control of the bacterial count without any preservatives, to make sure that the temperature condition in the refrigerator did not affect the normal growth of the bacteria (Del Rio *et al.,* 2007)

In our study we observed that dipping the chicken skins for 30 s before the inoculation with the bacteria and then dipping in sterile distilled water for 30 s to wash off the risks from the presence of chemical residues of TSP can reduce the *Campylobacter* count to 4 log in the first hour of application even with a 2% TSP. Whereas dipping the chicken skin for 30 seconds after the inoculation and then dipping in sterile distilled water for 30 seconds reduced the campylobacter count to 2-3 log.

The daily limit of TSP recommended being 70mg in total for a human being. TSP consumed as part of a Western junk food diet sometimes reached upwards of 500mg, which leads to an increased risk of developing osteoporosis (Kmiec, *et al* 2013). Therefore, we utilized only the combination of citric preservatives and we could see that this combination worked with a concentration of 0.3 % for Citrosan and 0.05% for Citrol-K- Ultra®. It showed a reduction of the bacterial count by 2 log in the first hours itself and the bacterial count came to a not detectable level by 48 h of the incubation time.

At this point it is very important to mention that according to Capita *et al*. (2003), TSP is much more effective in the skin models than the whole chicken pieces, because the wrinkles and skin irregularities of the whole chicken can give a full or partial protection to the bacteria, thus it is more difficult the removal of the bacterias.

Throughout the project, we utilized a high dose of the bacterial inoculum, which is $1.5X10^6$CFU/ml. This is done to simulate the worst-case scenario and to prevent the interference of the accompanying bacterial flora.

By analyzing statistically using the method Scheffe all the combination treatments with the preservatives, showed no significant difference among the different treatment with the bacterial strains ($p \leq 0.05$). This means, all the treatments are effective against the growth of the bacterial strain *Campylobacter jejuni* in the food model.

A study by Silvan *et al*., in 2012, showed the antimicrobial activity of a grape seed extract against different strains of *Campylobacter*. The growth inhibition was in the range of 5.08 – 6.97 log CFU/ml in 24 hours of the treatment demonstrated the strong capacity of the Grape Seed Extract to inhibit the *Campylobacte*r growth. Another study done by Zakariene et al, in 2015 with spice based marinated against *Campylobacter* on fresh broiler chicken wings. They used 6 different marinated which contain spices like thyme, rosemary, basil, marjoram, black pepper, sweet red pepper and chemical additives monosodium glutamate, sodium diacetate, calcium lactate and also bioactive compounds like linalool, cinnamaldehyde, lactic acid. Their study showed that the thyme based marinate was more effective against *Campylobacter jejuni* by a reduction count of 1.04 log CFU/g during storage for 168 h at 4°C temperature,

Sensory evaluations have been defined as a scientific discipline used to evoke, measure, analyze and interpret those responses to products as perceived through the senses of sight, smell, touch, taste and hearing (Dimple and Rohanie 2013). Sensory quality is the ultimate measure of product quality and success. Sensory analysis comprises a variety of powerful and sensitive tools to measure human responses to foods and other products. Selection of the appropriate test, test conditions, and data analysis result in reproducible, powerful and relevant results (Drake *et al* 2007)**.**

Sensory analysis can be consider as an interdisciplinary science that uses human panelists sensory perception related to thresholds of determination of attributes, the variance in individual sensory response experimental design to measure the sensory characteristics and the

acceptability of food products, as well as many other materials. Since there is, no one instrument that can replicate or replace the human psychological and emotional response, the sensory evaluation component of any food study is essential and importance of good experimental design cannot be overemphasize in sensory experiments (Dimple and Rohanie 2013).

There are many types of sensory analysis methods, the most popular being difference tests, descriptive analysis and consumer acceptance testing. Difference tests include the triangle test, where the panel member attempts to detect which one of the 3 samples is different from the other two, and duo-trio tests, where the panel member selects which one of the 2 samples is different from the identified standard. Descriptive sensory analysis uses several techniques that seek to discriminate between ranges of products based on their sensory characteristics and to determine a qualitative description of the sensory differences that can be identify, not just the defects. Consumer acceptance, preference, and hedonic (degree of liking) tests are used to determine the degree of consumer acceptance for a product. It is also considered as consumer tests since they should be conducted using untrained consumer panels. Although panelists can be asked to indicate their degree of liking, preference or acceptance of a product directly, hedonic tests are often used to measure preference or acceptance indirectly. Category scales, ranking tests and the paired-comparison test can all be used to assess product acceptance. Acceptance of a food product usually indicates actual use of the product purchase and eating. (Dimple and Rohanie 2013).

Whole chicken carcass treated with TSP, dodecahydrate were found to be pinker in appearance compared to the untreated control and were preferred by the untrained panelists. (Samant *et al.,* 2015). Based on the panelist's poll in the sensory analysis of our study, the results showed that the treatment with 2%TSP, 0.3% Citrosan and 0.05% Citrol-K-Ultra® was accepted more in the aspect of color.

Among the organic acids, citric acid treatments have been observe to have varying results on the odor /flavor characteristics of raw, cut-up poultry (Samant *et al.,* 2015). In our study according the panelists poll, the treatment with only Citrosan and Citrol-K-Ultra® was agree in terms of flavor.

The impact of antimicrobials on the texture characteristics of poultry meat products is not been studied as extensively as other sensory aspects (Samant *et al.,* 2015).Our study showed that

comparing with the treatment without any preservatives, the treatment with TSP was more accepted in terms of texture. This could be the usage of TSP, which in some poultry industry used as a humidifying agent. Further studies are necessary to clarify this point. All the other variables like taste and general acceptance showed no difference with respect to the control. All these treatments analyzed statistically with Chi-square method and showed no significant difference ($p \leq 0.05$).

The purpose of the sensory analysis was to check if these preservative in combination would be having any change in the organoleptic properties of the chicken meat. Since the consumers are so much worried about the usage of chemical preservatives the food scientist are working on reducing the concentration of synthetic preservatives or utilizing more preservatives that are natural. Therefore, it is important to do more investigation in this area.

Finally, we can say that the hypothesis that we planted at the beginning of this project is accept since the preservatives in combinations can reduce the concentration of each of them, but still maintain its antimicrobial activity in an effective way without affecting the organoleptic properties of the chicken meat.

CONCLUSIONS

The Minimal Bactericidal concentration (MBC) of TSP, Citrosan and Citrol-K-Ultra® against the growth of *Campylobacter jejuni* in vitro was 0.5%, 0.05% and 0.0006% respectively.

The MBC of the preservatives in combination: 1) TSP-Citrosan was 0.4 and 0.03% respectively, 2) TSP-Citrol-K-Ultra® was 0.3 and 0.0003% respectively and 3) Citrosan-Citrol-K-Ultra® was 0.04 and 0.0005% respectively.

The effective lowest final concentrations that utilized in the chicken model were TSP 2%, Citrosan 0.3% and Citrol 0.05% show a reduction of *Campylobacter* count to 4 log in the first hour and a complete reduction of the bacterial count by 48 h of incubation.

Application of the 2% TSP before the bacterial inoculation to the chicken skin and then apply the Citrosan-Citrol-K-Ultra® showed a reduction of the *Campylobacter* count to 4 log in the first hour and to a non detectable level (<100 cell/ml)

The final lowest concentration and their combinations of the preservative don't have made any organoleptic changes to the chicken wings.

Perspective

The search for antimicrobial agents that eliminate the pathogens in food is an issue that is still in development, not only to check for the isolated microorganisms, but also its real presence, that is the presence of other microorganisms or the possibility to form biofilms either in food matrices or the equipments used in the food industry.

Likewise, the development of an alternative state of certain microorganisms, currently known as VBNC state make an urgent necessity research in this for the antimicrobial compounds either alone or in combination to minimize the risks that this bacterial state can cause in the food industry.

There is too much to be done in the research for antimicrobial compounds, in addition to taking into account that the people now are more concerned about the safe and organic foods that does not damage the health due to the chemical used in the foods.

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