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RESEARCH ARTICLE

Eugenol, citral, and hexanal, alone or in combination with heat, affect viability, biofilm formation, and swarming on Shiga-toxin-producing *Escherichia coli*

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Abstract Shiga-toxin-producing *Escherichia coli* strains are pathogenic for humans and cause mild to severe illnesses. In this study, the antimicrobial effect of citral, eugenol, and hexanal in combination with heat shock (HS) was evaluated in terms of the growth, biofilm formation, swarming, and expression of virulence genes of STEC serotypes (O157:H7, O103, O111, and O26). Eugenol was the most effective compound against the growth of *E. coli* strains (MBC = 0.58 to 0.73 mg/mL), followed by citral (MBC = 0.86 to 1.26 mg/mL) and hexanal (MBC = 2.24 to 2.52 mg/mL). Biofilm formation and swarming motility have great variability between STEC strains. Natural compounds—alone or combined with HS—inhibited biofilm formation; however, swarming motility was induced by most treatments. The expression of the studied genes during biofilm formation and swarming under natural antimicrobials was affected but not in a uniform pattern. These treatments could be used to control contamination of STEC and inhibit biofilm formation.

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Introduction

The pathogenic strains of *Escherichia coli* have been grouped into at least six pathotypes based on epidemiological evidence, phenotypic traits, clinical features, and specific virulence factors. One of them is Shiga-toxin *E. coli* (STEC), responsible for pathological processes such as hemorrhagic colitis (HC) and serious complications (hemolytic uremic syndrome, HUS) (Page and Liles, 2013). The serotype O157:H7 belongs to this group and is the most frequently reported to cause diseases (Rangel et al., 2005). However, other serotypes, named O26, O45, O103, O111, O121, and O145, known as "big-six," are also very frequent and are responsible for 70% of the 1579 illness episodes, and 202 and hospitalizations for non-O157 STEC-related foodborne illness in the United States each year (Page and Liles, 2013).

Although Shiga toxins (Stx 1 and/or 2) are the most important virulence factors of these bacteria, other processes, such as swarming motility and biofilm formation, also contribute to the development or increase of their persistence in environments. Swarming motility allows bacteria to migrate cooperatively over semi-solid and solid surfaces and increases resistance to antimicrobials (Partridge et al., 2019). Biofilms are sessile microbial communities on surfaces formed through the secretion of extracellular polymeric substances. These compounds enhance adherence to surfaces and induce microbial aggregation (Donlan and Costerton, 2002). The flagellum is primarily a motility organelle that enables movement and chemotaxis. In addition to motility, a flagellum can

participate in biofilm formation, protein export, and adhesion (Haiko and Westerlund-Wikström, 2013). In humans, STEC pathogenesis involves the adhesion of bacteria to the ileum epithelium, followed by colon colonization and production of Stx, thereby impairing epithelial barrier function and ion transport, causing diarrhea. Stx may reach the bloodstream and be disseminated to extra-intestinal tissues, producing more severe diseases and important sequelae such as HUS that may result in death (Montero et al., 2017).

The increase in antibiotic resistance among microbial pathogens is accelerating the search for alternatives to control their presence in foods and bacterial infections. Plant-based antimicrobials are believed to be less likely to induce bacterial resistance, and many of them are classified as generally safe for human consumption (Kim et al., 2016). Extracts of *Lippia graveolens* and *Haematoxylon brassiletto*, and compounds such as carvacrol, brazilin, and citral, are bactericidal against enterohemorrhagic *E. coli* O157:H7, enteroaggregative *E. coli* strain 042, and a strain of the O104:H4 serotype. Most of these compounds reduce swarming motility by 7–100%, except for carvacrol, which promotes motility in two strains. Various concentrations of compounds induced biofilm formation; however, most compounds decreased *stx2* expression (García-Heredia et al., 2016). The phenolic compounds tannic acid, gallic acid, methyl gallate, and epigallocatechin gallate are bactericidal against enteropathogenic *E. coli,* enterohemorrhagic *E. coli*, and enterotoxigenic *E. coli*. Lower concentrations than bactericidal decrease swarming motility (14.8–100%). Gallic acid reduces biofilm formation; however, tannic acid, methyl gallate, and epigallocatechin gallate induce biofilm formation in some strains at specific concentrations. Tannic acid induces the overexpression of *csgA*, *csgD*, and *cyaA*, whereas the other compounds do not have effects or reduce their expression (Dávila-Aviña et al., 2020).

Citral, eugenol, and hexanal are attractive compounds to be analyzed for the inhibition of Shiga-toxin-producing *E. coli*. Citral is a major active component of citrus oils and is commonly added to foods and beverages (e.g., soft drinks and desserts) and is approved for use in foods by the U.S. Food and Drug Administration (FDA, GRAS, 21 CFR 182·60). In addition, citral has shown antibacterial activity against *E. coli*, *Salmonella*, and *Listeria monocytogenes* (Shi et al., 2017). Eugenol is used as a flavoring agent in foods and has demonstrated antimicrobial properties, such as inhibiting biofilm and reducing toxin-related gene expression (Mak et al., 2019). Hexanal possesses a noticeable activity against several fungi and Gram-positive and Gram-negative bacteria, being a good candidate to be explored for its activity against STEC strains (Patrignani et al., 2008).

Bacteria have developed mechanisms to survive in unfavorable conditions. This is a response to stress that results in a modified pattern of gene expression that generally results in increased tolerance to the stress that triggered the response, and usually to many other stressors as well (Pichereau et al., 2000). Heat has been a common method to reduce or kill the microbial population (Van Impe et al., 2018); few studies have dealt with the combined effect of natural antimicrobials and heat stress on the expression of virulence factors of pathogenic *E. coli*. Therefore, the present study aimed to evaluate the effect of citral, eugenol, and hexanal in individual form and combined with heat on viability, virulence factors, and virulence-associated gene expression on STEC serotypes.

Material and methods

Antimicrobial agents

Citral (purity ≥ 96%, #W230316-SAMPLE-K, Lot: MKBH9971V), eugenol (purity ≥ 99%, #E51791-100 g, Lot: MKBJ0904V), and hexanal (purity ≥ 95%, #W2255726-25 g-K, Lot: MKBJ9990V) were obtained from Sigma-Aldrich (Mexico). The stock solutions were prepared in 1% (v/v) tween 80 (Hycel, Mexico) and stored in dark flasks. Solutions were always prepared and used the same day. They were filter-sterilized (0.45 µm) prior to use.

Bacterial strains and growth conditions

STEC serotype O26 was provided by Dr. Lee Ann Jaykus of North Carolina State University, STEC O103 was obtained from the *E. coli* Reference Center at Pennsylvania State University, STEC O111: MN (ATCC 43887), which was donated by Dr. Fernando Navarro (CINVESTAV, Mexico), and the EHEC O157: H7 strain (ATCC 43895) and non-pathogenic strain *E. coli* (NPEC) ATCC 25922 were provided by Dr. Lynn McLasborougth, Department of Food Sciences, University of Massachusetts, Amherst MA, USA. All strains were stored as stock cultures at − 80 °C in brain heart infusion broth (BHI, Bioxon, Mexico) with 20% (v/v) glycerol (Jalmek, Mexico). An aliquot of the stock culture was inoculated into BHI and incubated at 37 °C/24 h. To obtain a working culture, a loopful was streaked onto Petri dishes containing BHI agar, incubated at 37 °C/24 h, and stored at 4 °C for no longer than 4 weeks.

For the assays of the effect of compounds against STEC virulence factors, the EHEC O157:H7 strain was used as positive, while NPEC was used a negative control of *stx*1.

Minimum bactericidal concentration (MBC)

The MBC was determined using the method described by Castillo et al. (2014) with some modifications. To obtain a fresh culture, an aliquot of the working culture was inoculated into tubes with 5 mL of Mueller Hinton (MH) broth (Difco, USA) and incubated at 37 °C/24 h. Ten μL (~ 1×10^6 CFU/mL) of this culture was inoculated into tubes containing 1 mL of MH broth and various concentrations of the natural compounds (0.1% to 0.9% for citral, 0.1% to 1% for eugenol, and 1% to 5% for hexanal). After incubation at 37 °C/24 h, bacterial survival (growth/absence of growth) was determined by plating 100 μL of the culture onto Petri dishes with MH agar and incubated at 37 °C/24 h. MBC was defined as the lowest concentration of antimicrobials that produced no growth on the MH agar. Sterile MH broth without antimicrobials was used as the control.

Heat shock

The assays were performed using the strains NPEC, O157:H7 and O26. All the three strains were separately activated in LB broth and incubated at 37 °C/18–24 h. Cultures were adjusted to ~ 1×10^6 CFU/mL in LB broth, and various concentrations of the antimicrobial agents were added (0.1% and 0.05% citral, 0.25% and 0.1% eugenol, and 0.5% and 0.25% hexanal). The cultures were incubated at 37 °C/6 h, and sublethal heat shock (46 °C/1 h) was applied, followed by a lethal heat shock (55 °C/1 h). Aliquots were taken across time (before sublethal shock [time 0 h] and every 20 min for a total of 2 h), and viable cell counts were performed by serial dilutions in 0.85% NaCl (Fermont, Mexico), which were plated onto Petri dishes with nutrient plate count agar (Bioxon, Mexico) and incubated at 37 °C/48 h.

Combined effect of compounds and heat shock on bacteria

Biofilm

An assay was performed following the methodology described by Wang et al. (2012) with modifications. Strains were grown overnight as described above (to reach a population of ~ 1×10^6 CFU/mL). The culture was diluted 1:100 with sterile LB broth, and 200μL were added to wells of sterile 96-well flat-bottom polystyrene plates (Costar, Corning, NY) containing the natural compounds (citral 0.1%, eugenol 0.25%, and hexanal 0.5%, final concentrations) diluted in LB broth. Plates were exposed to sublethal and lethal heat shocks (as described above) and placed onto air-incubators. Then they were incubated at 37 °C for up to 24 h. Supernatants in each well were removed by aspiration, and the absorbance (A_{630nm}) of bacteria that adhered to the plate was measured using a microplate reader (Epoch, USA). Plate wells were washed with 200 μL of sterile saline phosphate buffer (PBS, pH 7.2) to remove the lightly bound cells. The wells were air-dried and stained with 100 μL of 0.1% crystal violet (CV) for 20 min at room temperature and then washed with PBS and allowed to dry. The CV that stained biofilms was dissolved with 100 μL of 85% ethanol, and the amount of CV extracted in each well was determined by measuring the A_{570nm}. The biofilm formation index (BFI) was determined as described by Naves et al. (2008) using the formula:

$$\mathrm{BFI} = \mathrm{AB} - \mathrm{CW}$$
$$\mathrm{BFI} = \mathrm{AB}/\mathrm{CW}$$
$$\mathrm{BFI} = (\mathrm{AB}-\mathrm{CW})/\mathrm{G}$$

where AB is the A_{570nm} of bacteria stained and adhered, CW is the A_{570nm} of control of LB medium (dye- and bacteria-free), BFI is the specific biofilm formation index, and G is the A_{630nm} of planktonic growth. Values of BFI $\geq$ 1.10 indicate strong (S) biofilm, while BFI = 0.70 – 1.09 indicates moderate (M) biofilm, BFI = 0.35 – 0.69 indicates weak (W) biofilm, and BFI $<$ 0.35 indicates non-forming (N) biofilm.

Swarming motility

Swarming motility was determined following the methodology described by Lee et al. (2013), with some modifications. Briefly, Petri dishes containing LB broth with 0.3% (w/v) bacteriological agar (Bioxon, Mexico) and antimicrobial agents (citral 0.1%, eugenol 0.25%, and hexanal 0.5%, final concentrations) were prepared. NPEC and O157:H7 and O26 strains were grown at 37 °C/16–18 h on LB broth. An aliquot (3 μL) of each strain was placed in a well previously made in the center of the LB semisolid agar plate. Plates were subjected to 46 °C/60 min (sublethal heat shock), followed by 55 °C/60 min (lethal shock). Plates without shock and/or without natural compounds were used as controls. All the plates were incubated at 37 °C/18–24 h.

Expression of virulence-related genes

Cells exposed to natural compounds and heat shock from biofilm and swarming assays were collected at about 18 h, and nucleic acid was extracted. The genes analyzed were related to biofilm formation, motility, heat shock, and Shiga Toxin 1 (which is the toxin produced by our isolates, Table 1). For RNA extraction, cells were washed with

Table 1 Target genes and primer sequences

Gene	Function	Sequence (5′- 3′)	References
csgD	Biofilm master regulator	F-CCGCTTGTGTCCGGTTTT	Chen et al. (2010)
		R- GAGATCGCTCGTTCGTTGTTC	
csgA	Codifies structural protein of the curli fimbrias	F- TGGCAGGTGTTGTTCCTCAGT	
		R- GTCAGAGTTACGGGCATCAGTTT	
flhD	Flagellar biosynthesis regulator	F -ACCTCCGAGTTGCTGAAACAC	Allison et al. (2012)
		R -TTGCTGGAGATCGTCAACGC	
flhC	Flagellar biosynthesis regulator	F- CGGCAGGATTCTGGGAAAGT	This study
		R- AGCGCCCGCTCCTCGTCAGC	
rpoH	Controls the thermal shock response	F- ATGACTGACAAAATGCAAAG	Hase et al. (2013)
		R- AGCGCCCGCTCCTCGTCAGC	
rpoE	Thermal shock and stress response in the membrane	F- ACGCCTGATAAGCGGTTGAA	This study
		R- ATGGCAATAACCTTGCGGGA	
rpoS	General stress regulator	F- TTCGTTTGCCGATTCACATC	Liu et al. (2010)
		R- TCTCTTCCGCACTTGGTTCA	
stx1	Shiga toxin 1	F-TGCATCGCTTTCATTTTTTTCA	Dowd (2007)
		R-CCACCTTTCCAGTTACACAATCAG	
GAPDH	Gliceraldehide-3-phosphate dehydrogenase (Housekeeping)	F- TCCGTGCTGCTCAGAAACG	Carey et al. (2008)
		R- CACTTTCTTCGCACCAGCG	

sterile PBS and suspended in TRIsure Reagent (Bioline, UK) following the manufacturer's instructions. RNA was converted to cDNA using the reverse transcription iScript™ cDNA Synthesis kit (Bio-Rad, Mexico).

Quantitative PCR (qPCR) was performed using a real-time PCR system (Pikoreal 96 Thermo Scientific, Finland) and iQ SYBR Green supermix (Bio-Rad, Mexico) with the following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 5 s, and 58 °C for 30 s. The GAPDH, a housekeeping gene, was used to normalize the levels of expression of the target genes detected. Expression levels of treated bacterial strains and control (untreated) were determined by measuring changes in fold expression using the ΔΔCq (Livak and Schmittgen, 2001).

Statistical analysis

All assays were performed in at least three independent assays and with three or more replicates. Comparisons between treatments were analyzed by ANOVA, and the Tukey–Kramer test was used for means comparisons using SPSS software version 23 (IBM Corp. Armonk, NY). The significance used was 95%.

Results and discussion

Effect of natural products on bacterial growth

All three natural products that were analyzed affected the bacterial growth (Table 2); eugenol showed the greatest ($p < 0.05$) inhibitory effect on the growth of the strains analyzed (MBC from 0.58 mg/mL to 0.73 mg/mL), followed by citral (MBC from 0.86 mg/mL to 1.26 mg/mL) and hexanal (2.24 mg/mL to 2.52 mg/mL). An interesting finding was that NPEC was the most sensitive strain. Pathogenic strains analyzed showed similarities between them, but the pathogenic strains were similar or more resistant than the non-pathogenic strain (Table 2).

Antimicrobials of plant origin have been proven to affect various bacterial processes. Eugenol is an essential oil extracted from cloves, known for its antioxidant capacity (Gill and Holley, 2006). In previous studies, eugenol showed bacteriostatic and bactericidal activity against Gram-negative bacteria (*E. coli,* and *Salmonella*) and Gram-positive bacteria (*Enterobacter aerogenes*, *Staphylococcus aureus*, and *Listeria monocytogenes*) (Gill and Holley, 2006; Mak et al., 2019). The antimicrobial properties of eugenol may be due to cell membrane disruption; eugenol is hydrophobic and interacts with the cell membrane, leading to leakage of intracellular components due to non-specific permeability (Gill and Holley, 2006). In our study, eugenol was the compound with the highest growth-

Table 2 Minimal bactericidal concentration (MBC mg/mL) of natural compounds for the growth of *E. coli* serotypes

Antimicrobial agents	MBC mg/mL				
	STEC O103	STEC O26	STEC O111	EHEC O157:H7	NPEC ATCC 25922
Citral	1.00 ± 0.03 *b*[1]	1.26 ± 0.05 *a*	0.86 ± 0.05 *b*	1.00 ± 0.07 *b*	0.94 ± 0.15 *b*
Eugenol	0.73 ± 0.02 *a*	0.71 ± 0.01 *a*	0.73 ± 0.02 *a*	0.66 ± 0.01 *b*	0.58 ± 0.04 *c*
Hexanal	2.24 ± 0.05 *b*	2.42 ± 0.08 *a*	2.24 ± 0.05 *b*	2.52 ± 0.11 *a*	2.24 ± 0.05 *b*

[1] ± standard deviation

Different letters represent significant differences ($p < 0.05$)

inhibitory effect on bacteria. Although Pei et al. (2009) reported MBC of 1.6 mg/mL for EHEC O157:H7 ATCC 43895, the MBC found in our study for all the strains was lower (0.58 – 0.73 mg/mL); these differences can be due to variations in the physiology of the serotypes isolates, culture media, and purity of chemical compounds.

The effectiveness of citral has been previously demonstrated against various bacteria including various pathotypes of *E. coli* EHEC serotype O157:H7 strain, EAEC 042, EAEC O104:H4 (MBC 0.12 to 0.20, mg/mL). The essential oil contains citral and other terpenes that cause damage to and rupture the bacterial cell membrane (Zhang et al., 2020).

Hexanal, a green-leaf-derived C6- aldehyde, is a component of the aroma of a large number of fruits, including apples, with antimicrobial and phospholipase D inhibition properties and is useful for post-harvest preservation of fresh fruits and produce. This compound has been reported to be almost inactive against *Escherichia coli* (ATCC 10538) (Bisignano et al., 2001). However, in our study, it exhibited an antimicrobial effect in the strains tested with an MBC ranging between 2.24–2.52 mg/mL. Hexanal was the compound with the highest MBC for the *E. coli* strains. The antimicrobial activity could be due to the blocking activity of superoxide dismutase (SOD); however, its effects seem to depend on the specific in-site properties (volatility and liposolubility) of hexanal and can change when cells are under oxidative stress (Matos et al., 2020).

Effect of natural compounds and heat shock on STEC virulence factors

Biofilm formation

Biofilms are surface-bound microbial communities with distinct properties that have a tremendous impact on public health and food security (Wang, 2019). Biofilms could be constituted of multiple species of microorganisms; complex interactions within the community influence the architecture, activity, and resistance to antimicrobials (Wang, 2019). The serotype O26 formed a strong biofilm and the NPEC ATCC 25922 formed a moderate biofilm. The serotypes O103, 0111, and O157:H7 did no form biofilms under the conditions analyzed (Fig. 1). The compounds analyzed were found to decrease the biofilm formation of serotype O26 and NPEC ATCC 25922. However, these did not produce significant changes on serotypes O103, 0111, and O157:H7, although hexanal slightly increased biofilm formation in serotype O111. HS alone did not produce major changes in the biofilm formation of the strains; it only reduced the biofilm formation of NPEC ATCC 25922. When compounds + HS were applied, they reduced biofilm formation on serotype O26 and NPEC ATCC 25922 and produced no change in the other strains.

These results are of interest to industry and health authorities because these antimicrobials could reduce all the benefits provided by biofilm formation and could reduce contamination problems and diseases related to these bacteria. It is important to note that many natural antimicrobials induce biofilm formation (García-Heredia et al., 2016), which makes them inappropriate for use in the control of microbial contamination.

Swarming motility

In most cases, compounds increased swarming motility, especially in the pathogenic strains tested; in these strains, when no motility was increased, it remained unchanged (no decrease was observed, Table 3). Only in the NPEC isolate was a reduction in swarming observed by eugenol and hexanal and swarming was unchanged by citral. Heat shock increased swarming in O103 and O26 reduced it in the NPEC, but it remained unchanged in O157:H7. When HS combined with compounds was applied, again, an increase or no change in swarming was observed for almost all cases. However, the NPEC isolate exhibited a swarming reduction by HS plus citral or hexanal.

The strain O111 was the isolate most affected by treatments by the natural products provoking an increase in motility ($p < 0.05$), whereas the strain O103 was affected only when the natural products were in combination with

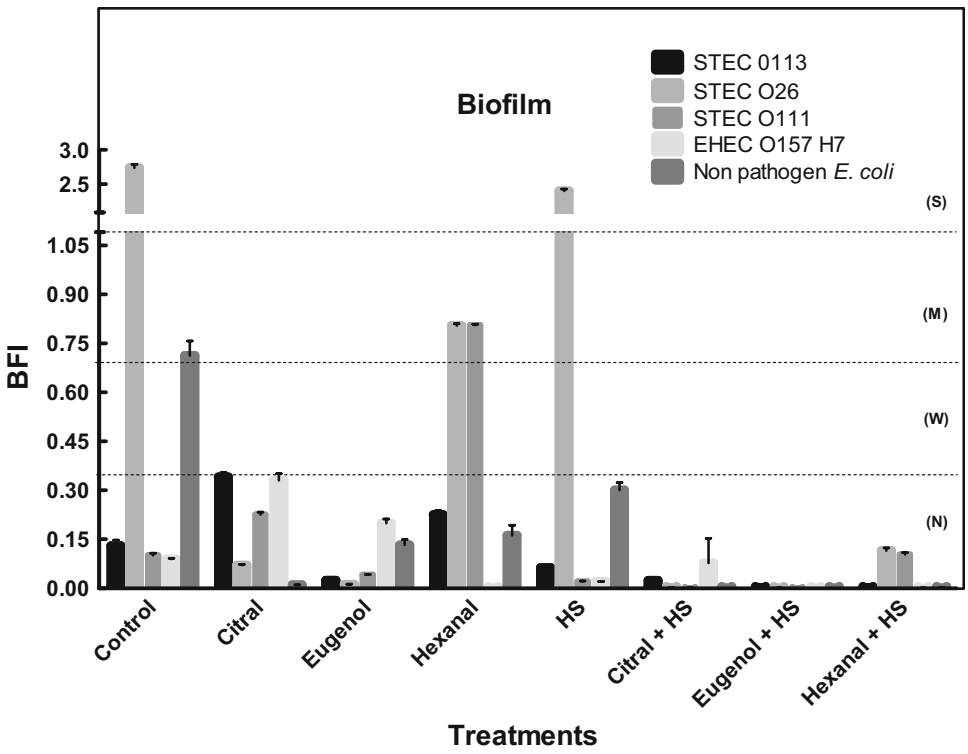

Fig. 1 Effect of natural compounds (0.1% citral, 0.25% eugenol and 0.5% hexanal) and heat shock on biofilm formation of *E. coli* serotypes. BFI = Biofilm formation index. N = Non-forming (BFI < 0.35), W = weak biofilm (BFI > 0.35 to < 0.69, M = moderate biofilm (BFI > 0.70 to < 1.09) and S = strong biofilm (BFI ≥ 1.10)

Table 3 Effect of natural compounds (0.1% citral, 0.25% eugenol and 0.5% hexanal) on the motility of *E. coli* serotypes

Strains	Swarming migration (cm)							
	Control	Citral	Eugenol	Hexanal	HS	Citral + HS	Eugenol + HS	Hexanal + HS
STEC O103	0.3 ± 0.05*a*[1]	0.3 ± 0.05*a*	0.3 ± 0.04*a*	0.3 ± 0.03*a*	0.8 ± 0.10*c*	0.7 ± 0.03*bc*	0.6 ± 0.10*b*	1.0 ± 0.05*d*
STEC O26	4.5 ± 0.12*a*	5.4 ± 0.13*b*	4.3 ± 0.09*a*	5.1 ± 0.09*b*	5.3 ± 0.02*b*	4.4 ± 0.09*a*	5.1 ± 0.36*b*	5.3 ± 0.06*b*
STEC O111	0.5 ± 0.06*a*	1.4 ± 0.18*bc*	1.4 ± 0.21*bc*	1.5 ± 0.10*c*	0.5 ± 0.03*a*	1.0 ± 0.15*b*	1.5 ± 0.11*bc*	1.3 ± 0.29*bc*
EHEC O157:H7	5.5 ± 0.21*b*	5.4 ± 0.07*b*	7.4 ± 0.27*c*	7.0 ± 0.08*c*	5.5 ± 0.16*b*	5.6 ± 0.08*b*	4.5 ± 0.11*a*	5.5 ± 0.27*b*
NPEC ATCC 25922	2.8 ± 0.05*e*	2.6 ± 0.05*de*	2.0 ± 0.29*abc*	1.9 ± 0.13*ab*	1.7 ± 0.09*a*	2.1 ± 0.06*bc*	2.4 ± 0.18*cde*	2.3 ± 0.08*bcd*

[1] ± standard deviation

Different letters represent significant differences ($p < 0.05$)

HS, increasing the swarming motility ($p < 0.05$). The treatment of eugenol + HS increased the motility in all the STEC (O103, O26, and O111) strains analyzed, but not in the EHEC (O157:H7) and NPEC strains. Indeed, the swarming motility for NPEC isolate, in most cases, was reduced or remained unchanged by the treatments, though no increase was observed.

Antibiotics may influence swimming and swarm motility in multi-drug-resistant *S.* Typhimurium (Brunelle et al., 2017), although the process by which these alterations are created is not known. The effect of plant compounds on swarming motility has been studied in EHEC O157:H7, EAEH 042, and *E. coli* serotype O104:H4 strains, demonstrating that carvacrol promoted the motility of two of the tested strains (García-Heredia et al., 2016). This effect is similar to that obtained in this study, where citral, eugenol, hexanal, and heat shock promote motility in STEC O26, and eugenol and hexanal in EHEC O157:H7.

Expression of biofilm and virulence-related genes

Because strain O26 was the only one that exhibited a response in inhibiting the biofilm and also showed an increase in swarming motility by exposure to natural

products, alone or in combination with HS, the assays of gene expression were performed only in this strain.

During biofilm assays, the expression of biofilm-related, heat shock, and Shiga toxin genes was analyzed (Table 4). Expression of biofilm-related genes (*csgA* and *csgD*) was mostly unaffected by the chemical compounds; however, when these compounds were combined with HS, overexpression was observed in almost all treatments. Analysis of the heat-shock-related genes indicates that chemical compounds and HS induce overexpression of *rpoE* in all cases, whereas no major changes were observed for the expression of *rpoH* under these conditions. The expression of *rpoS* remained unchanged by the effect of chemical compounds; however, HS, eugenol + HS, and hexanal + HS slightly overexpressed it, while citral + HS provoked no major changes (Table 4). The expression of the Shiga toxin gene (*stx1*) was underexpressed by eugenol but overexpressed by citral + HS and eugenol + HS.

Expression of flagella, HS, and Shiga toxin genes was analyzed during swarming motility assays (Table 4). *flhC* was underexpressed by citral and hexanal but overexpressed by eugenol. HS alone or in combination with the three compounds also overexpressed this gene. *flhD* and the HS-related genes did not show a homogeneous pattern of expression. In most cases, *stx1* was overexpressed, especially under the effect of eugenol and hexanal alone or in combination with HS. HS alone provoked overexpression of all the genes studied. Eugenol alone or when combined with HS also provoked an overexpression of most genes (Table 4).

Although plant compounds reduced the expression of biofilm formation, they did not have a major effect on the expression of genes *csgA* and *csgD*, which are related to fimbria and curli and biofilm regulation, respectively. Their expression increased when the compounds were combined with heat shock but neither increased biofilm. Heat-shock-related genes behave differently under exposure of *E. coli* to the treatments; *rpoE* is a gene involved in stress response specifically at the membrane level, and its expression increased by exposure to all the treatments. On the other hand, the expression of *rpoH* remained unchanged and *rpoS* remained unchanged by the chemical compounds alone, though heat shock alone or combined with compounds (except with citral) increased its expression. These results suggest that bacteria were stressed by these natural compounds but were unable to induce biofilm formation under these conditions.

Shiga toxin is considered to be a very important STEC virulence factor. Epigallocatechin gallate and citric acid decreased *stx1* gene expression of *E. coli* O157:H7 (Yang et al., 2018). Treatment with cinnamon bark oil reduced EHEC *stx* gene expression; the effect observed with this essential oil could be attributed to eugenol (Kim et al., 2015). In our study, during biofilm formation, natural products and HS alone reduced or did not change its expression. However, citral and eugenol, when combined with HS, overexpressed *stx1*.

In general, swarming bacteria exhibit more extended runs and higher speeds than bacteria swimming individually in a liquid medium. Swarming is facilitated by several

Table 4 Effect of natural compounds (0.1% citral, 0.25% eugenol and 0.5% hexanal) on expression of genes related to biofilm formation and swarming motility on STEC O26

Genes	Control[1]	Gene expression (fold-expression related to control)						
		Citral	Eugenol	Hexanal	HS	Citral + HS	Eugenol + HS	Hexanal + HS
Biofilm								
csgA	1.0 ± 0.0*bc*	0.2 ± 0.1*a*	0.7 ± 0.0*ab*	1.4 ± 0.3*c*	5.5 ± 0.4*e*	3.7 ± 0.1*d*	3.7 ± 0.1*d*	0.8 ± 0.1*b*
csgD	1.0 ± 0.0*ab*	0.8 ± 0.1*a*	0.7 ± 0.0*a*	1.0 ± 0.3*ab*	1.3 ± 0.1*b*	2.1 ± 0.1*c*	4.5 ± 0.0*d*	1.9 ± 0.3*c*
rpoE	1.0 ± 0.0*a*	2.9 ± 0.2*c*	3.0 ± 0.1*c*	4.9 ± 0.1*d*	2.0 ± 0.3*b*	2.5 ± 0.4*bc*	5.8 ± 0.1*e*	2.6 ± 0.4*bc*
rpoH	1.0 ± 0.0*abc*	0.5 ± 0.2*a*	1.5 ± 0.3*bc*	0.8 ± 0.4*ab*	1.6 ± 0.0*bc*	1.7 ± 0.3*c*	1.5 ± 0.3*bc*	1.4 ± 0.4*bc*
rpoS	1.0 ± 0.0*bc*	0.2 ± 0.0*a*	0.1 ± 0.0*a*	0.1 ± 0.0*a*	1.3 ± 0.3*c*	0.8 ± 0.1*b*	2.1 ± 0.0*e*	1.8 ± 0.2*d*
Stx1	1.0 ± 0.0*ab*	1.3 ± 0.2*ab*	0.3 ± 0.0*a*	0.5 ± 0.0*ab*	1.4 ± 0.5*b*	2.8 ± 0.9*c*	3.3 ± 0.3*c*	1.4 ± 0.2*ab*
Swarming								
flhC	1.0 ± 0.0*ab*	0.7 ± 0.1*a*	4.5 ± 0.4*f*	0.4 ± 0.0*a*	2.5 ± 0.2*e*	1.7 ± 0.1 *cd*	1.5 ± 0.4*bc*	2.2 ± 0.1*de*
flhD	1.0 ± 0.0*b*	1.0 ± 0.0*b*	1.4 ± 0.1*d*	0.2 ± 0.0*a*	2.0 ± 0.0*e*	1.0 ± 0.0*b*	1.1 ± 0.1*c*	0.2 ± 0.0*a*
rpoE	1.0 ± 0.0*b*	1.8 ± 0.0*c*	1.7 ± 0.2*c*	1.0 ± 0.0*b*	2.9 ± 0.2*d*	0.8 ± 0.1*b*	0.8 ± 0.1*b*	0.3 ± 0.0*a*
rpoH	1.0 ± 0.0*bcd*	1.1 ± 0.0*d*	1.1 ± 0.0 *cd*	0.8 ± 0.0*abc*	1.6 ± 0.1*e*	1.0 ± 0.3*bcd*	0.6 ± 0.1*a*	0.7 ± 0.1*ab*
rpoS	1.0 ± 0.0*b*	1.7 ± 0.0*d*	1.8 ± 0.1*d*	2.2 ± 0.1*e*	1.3 ± 0.0*c*	0.5 ± 0.0*a*	1.2 ± 0.0*bc*	1.2 ± 0.0*c*
stx1	1.0 ± 0.0*b*	1.1 ± 0.1*b*	1.4 ± 0.1 *cd*	1.7 ± 0.0*d*	1.3 ± 0.1*bc*	0.2 ± 0.0*a*	2.3 ± 0.3*e*	1.3 ± 0.1*bc*

[1]All genes were normalized (GAPDH). Different letters represent significant differences ($p < 0.05$)

alterations, such as increased flagellum numbers and special stator proteins (MotA and MotB, or secreted surfactants), and is associated with antimicrobial resistance (Partridge et al., 2019). The inhibitory effect of various essential oils, such as carvacrol, on swarming motility has been reported in *E. coli* strains (serotypes O157:H7, 042, and O104:H4) (García-Heredia et al., 2016). In our study, this inhibitory effect was observed mainly in the NPEC strain in most of the treatments studied. However, most of the treatments applied to the pathogenic strains provoked stimulation of swarming motility. The promotion of swarming motility by antibiotics has been observed in multi-drug-resistant *S.* Typhimurium, indicating that many factors can modify the motility patterns, such as antibiotic type, concentration, and resistance pattern. Identification of these factors can help create an understanding of how external compounds can influence bacteria (Brunelle et al., 2017).

The expression of genes during swarming motility exhibits different behaviors. *flhC*, a flagella-related gene, exhibits variable behavior under exposure of the compounds; however, HS alone or when combined with compounds increased its expression. *flhD*, another flagella-related gene, also exhibits variable expression under exposure of compounds, while HS overexpresses it, although not when combined with compounds. The heat-shock-related *rpoE* gene was overexpressed by citral and eugenol and HS, however, when citral and eugenol were combined with HS, no major effect was observed. Although compounds and HS overexpressed *rpoS*, when combined with the antimicrobials, an underexpression or no effect was observed. With this result, we cannot observe a constant behavior between treatments and gene expression. However, it was interesting to observe that HS alone overexpressed all studied genes during increased biofilm formation and increased swarming motility. As it is known, *E. coli* reacts to heat mainly through gene regulations, including the induction of heat shock proteins, and factors σ^E and σ^S, to re-fold misfolded proteins, and survive heat stress (Li and Gänzle, 2016). Heat treatment is a common intervention for reducing or eliminating bacterial cells.

In conclusion, eugenol, citral, and hexanal showed bactericidal activity against STEC strains O103, O111, and O26. Our study demonstrated that biofilm formation and swarming motility have great variability between STEC strains. Natural compounds, alone or combined with HS, inhibited biofilm formation; however, most treatments induced swarming motility. The expression of the studied genes during biofilm formation and swarming under natural antimicrobials was affected but not in a uniform pattern. This study shows that citral, eugenol, and hexanal, when applied at proper concentrations, could be used alone or in combination with HS to kill or reduce the survival of STEC and inhibit biofilm formation. Future studies would be aimed at determining the efficacy of these treatments on STEC isolates in heat-processed foods.

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Compliance with ethical standards

Conflict of interest We declare no conflict of interest.

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