FEMS Microbiology Letters, 362, 2015, 1–7

doi: 10.1093/femsle/fnu021
Advance Access Publication Date: 4 December 2014
Research letter

RESEARCH LETTER – Pathogens & Pathogenicity

The impact of oregano (*Origanum heracleoticum*) essential oil and carvacrol on virulence gene transcription by *Escherichia coli* O157:H7

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One Sentence Summary: Impact of oregano essential oil and carvacrol on virulence gene transcription of Escherichia coli O157:H7.
Editor: Séamus Fanning

ABSTRACT

The aim of the current study was to determine, via reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis, the effect of oregano essential oil (*Origanum heracleoticum*) and carvacrol, its major component, on the expression of virulence-associated genes in enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 ATCC strain 35150. Both oregano oil and carvacrol demonstrated their efficacy firstly, by inhibiting the transcription of the *ler* gene involved in upregulation of the LEE2, LEE3 and LEE4 promoters and of attaching and effacing lesions and secondly by decreasing both Shiga toxin and fliC genes expression. In addition, a decrease in *luxS* gene transcription involved in quorum sensing was observed. These results were dose dependent and showed a specific effect of *O. heracleoticum* and carvacrol in downregulating the expression of virulence genes in EHEC O157:H7. These findings suggest that oregano oil and carvacrol have the potential to mitigate the adverse health effects caused by virulence gene expression in EHEC O157:H7, through the use of these substances as natural antibacterial additives in foods or as an alternative to antibiotics.

Key words: Oregano essential oil; carvacrol; EHEC O157:H7; virulence gene expression

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 was first recognized as a food pathogen in 1982 (Paton and Paton 1998). Since then, this organism has been implicated in sporadic cases and outbreaks of enterohaemorrhagic diarrhea worldwide (EFSA and ECDC 2013; Crim *et al.*, 2014). EHEC O157:H7 is a member of the attaching and effacing (A/E) *E. coli* group. It possesses virulence factors essential for adhesion of the pathogen to intestinal epithelial cells (attachment) and is responsible for the destruction of the brush border of microvilli (effacement) (Paton and Paton 1998). This pathogen possesses a chromosomal pathogenicity island, termed as the locus of enterocyte effacement (LEE). Regulation of LEE has also been reported to be influenced by cell-to-cell communication, also called quorum

Received: 10 June 2014; **Accepted:** 5 November 2014

sensing (QS; Xavier and Bassler 2003). The gene *luxS*, responsible for QS regulation, is an important tool for EHEC O157:H7 in regulating the gene expression of some virulence factors such as type III-secretion (LEE operon) and bacterial motility (*fliC*) (Xavier and Bassler 2003; Medellin-Peña *et al.*, 2007).

The bacteria belonging to the Shiga-toxin-producing group of *E. coli* (STEC) are important pathogens that can cause gastrointestinal infections in humans. Production of Shiga toxin (Stx) may cause local damage to blood vessels in the colon and result in bloody diarrhea (Paton and Paton 1998). If sufficient Stx is absorbed into the bloodstream, vascular endothelial sites rich in toxin receptors will become damaged, leading to impaired function. In such cases, resulting chronic kidney damage and neurological disorders are observed, such as haemolytic uremic syndrome (HUS), due to the action of the two most important members of the Stx family, Stx1 and Stx2 (Mainil and Daube 2005). STEC are capable of producing either Stx1 or Stx2, or both; however, it is Stx2 that is associated with the more severe disease in humans. Stx2 has been reported to induce a multifaceted host inflammatory response (Paton and Paton 1998). Several studies have been carried out on different Stx2 variants as well as on its structure (Bertin *et al.*, 2001; Fraser *et al.*, 2004). Stx2 is known to consist of one A-subunit associated with five B-subunits. The mechanism of HUS manifestation has been reported to be caused by the successive binding of B-subunits to globotriaosylceramide followed by A-subunit inactivation of the 60 S ribosomal subunit, resulting in inhibition of protein synthesis (Fraser *et al.*, 2004; Robins-Browne 2005). For treatment of HUS in cases of infection, Mukherjee *et al.* (2002) suggested the potential use of specific monoclonal antibodies (HuMABs) to neutralize Stx2.

However, there is an urgent need, in both the food and the pharmaceutical industries, to develop more potent antimicrobial agents in order to control these bacterial pathogens and virulence toxins. A considerable amount of research into extracts from herbs and spices, particularly their essential oils and naturally occurring compounds, has been gaining popularity with many researchers in terms of the potential antimicrobial activity of these substances against a wide range of microorganisms (Burt 2004; Oussalah *et al.*, 2007; Mith *et al.*, 2014). Oregano essential oil and its main component carvacrol (CA), classified as generally recognized as safe (GRAS) by US Food and Drug Administration, present a broad spectrum of antimicrobial activity against a wide range of microorganisms (Dorman and Deans 2000; Burt 2004; Rivas *et al.*, 2010; Castilho *et al.*, 2012; McDonnell *et al.*, 2012; Mith *et al.*, 2014). Oregano (*O. vulgare*) oil has been shown to render the bacterial cell membrane of *E. coli* O157:H7 permeable by causing the loss of cell contents after treatment (Burt and Reinders 2003). In addition, oregano (*O. compactum*) oil has been shown to induce membrane damage in *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Bouhdid *et al.*, 2009). The antimicrobial effects of those oregano oils were reported to be associated with their main bioactive component, CA. The mechanisms of antimicrobial activity have been described in previous studies (Helander *et al.*, 1998; Lambert *et al.*, 2001; Gill and Holley 2006; Xu *et al.*, 2008). However, the effects of oregano oil and CA on the expression of virulence genes have been little described. The aim of the present study was therefore to explore the ability of *O. heracleoticum* (OH) essential oil and its main constituent, CA, at subinhibitory concentrations (subMICs) to interfere with gene regulation involved in virulence expression in EHEC O157:H7.

Table 1. Major constituents of *O. heracleoticum* essential oil.

No.	RT (min)	Composition[a]	PA (%)
1	18.7	γ-Terpinene	6.06
2	20.1	p-Cymene	4.69
3	41.5	β-caryophellene	2.45
4	72.7	Thymol	7.47
5	74.3	CA	68.14

RT: retention time; PA: peak area.
[a]Based on data from gas chromatography analysis provided by the manufacturer, Pranarom.

MATERIALS AND METHODS

Samples and bacteria

OH essential oil was provided by Pranarom International (Ghislenghien, Belgium). The composition of the oregano essential oil used, as determined by gas chromatography, is shown in Table 1. CA (≥98%) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). EHEC O157:H7 ATCC 35150 (containing *eaeA*, *stx1* and *stx2*) was used in this study. The strain was stored at −80°C and subcultured on plate count agar at 37°C for 24 h prior to use.

To investigate the effect of oregano essential oil and its main component, CA, on gene transcription in *E. coli* O157:H7, reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis was conducted on the expression of the genes *ler*, *stx2B*, *fliC* and *luxS* (Table 2). Based on the recent findings of Mith *et al.* (2014) with regard to the minimum inhibitory concentrations (MICs) of OH [0.025% (v/v)] and CA [0.013% (v/v)], the subMICs of 0.005% (OH1 and CA1) and 0.008% (OH2 and CA2) were chosen for testing in this study. EHEC O157:H7 with an initial concentration of approximately 10^7 colony-forming units mL^{-1} was grown in brain–heart infusion (BHI) medium alone or in the presence of different subMICs of OH or CA. The sample was vortexed and then incubated at 37°C for 4 h with shaking at 250 rpm. Appropriate volumes of essential oils of OH and CA were added directly to the media without any solvent before doing the dilutions. Bacterial growth was determined by optical density (OD) measurement at 600 nm.

RNA purification and cDNA synthesis

The method of analysis used was adapted from Tellez *et al.* (2012) with some modifications. After 4 h incubation, cells were collected by centrifugation at 5000 rpm for 10 min (Eppendorf Centrifuge 5804, Hamburg, Germany) at room temperature. Cells were lysed using the RNeasy Mini Kit (Qiagen, Antwerp, Belgium) and samples were stored overnight at −80°C according to the manufacturer's instructions. On the following day, RNA was extracted using the same kit. After RNA extraction, DNA contamination was eliminated from each sample using the DNase I Recombinant RNase-Free Kit (Roche Diagnostics GmbH, Mannheim, Germany). A volume of 25 μL of total RNA was incubated at 37°C for 30 min with 1 μL of DNase I, 5 μL of incubation buffer and 19 μL of molecular grade water in a total volume of 50 μL. RNA purification was then carried out using the RNeasy MinElute Clean-up Kit (Qiagen, Antwerp, Belgium) and the sample was then solubilized in 25 μL of molecular grade water (Qiagen, Antwerp, Belgium). The quantity of RNA present was determined by measuring the absorbance at 260 nm using a NanoDrop 2000

Table 2. Primers used for RT-qPCR.

Primer	Sequence of PCR primers (5′–3′)[a]	Function	References
gst	F: CTTTGCCGTTAACCCTAAGGG R: GCTGCAATGTGCTCTAACCC	Housekeeping gene	Pfaffl (2001)
gnd	F: GGTAATACCTTCTTCCAGGACACC R: TAGTGCGCCCTCCTCACC	Housekeeping gene	Rashid *et al.* (2006)
ler	F: TTTCTTCTTCAGTGTCCTTCA R: TGCGGAGATTATTTATTATGA	Involved in A/E lesions	Medellin-Peña *et al.* (2007)
stx2B	F: AGATGTTTATGGCGGTTTTA R: TTAAACTGCACTTCAGCAAA	Encoding subunit B of stx2	Medellin-Peña *et al.* (2007)
fliC	F: TACCATCGCAAAAGCAACTCC R: GTCGGCAACGTTAGTGATACC	Motility	Medellin-Peña *et al.* (2007)
luxS	F: GATCATACCCGGATGGAAG R: AGAATGCTACGCGCAATATC	Involved in QS and autoinducer-2 synthesis	Medellin-Peña *et al.* (2007)

[a]F: forward; R: reverse.

Spectrophotometer (Thermo Scientific, USA). The quality of the RNA was verified by measuring the ratio of the absorbance at 260 nm/280 nm and by agarose gel electrophoresis (Eurogentec, Seraing, Belgium). The RNA was heat-denatured (at 85°C for 5 min) before undergoing 2% (w/v) agarose gel electrophoresis (Eurogentec, Seraing, Belgium). The purified RNA was immediately used for reverse transcription-polymerase chain reaction (RT-PCR) using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Ghent, Belgium). Briefly, 0.5 μg of RNA was reverse transcribed with 0.8 μL of deoxyribonucleoside triphosphate (dNTP; 100 mM), 1 μL of Multiscribe Reverse Transcriptase (50 U μL^{-1}), 2 μL of 10X RT Random Primers and 2 μL of 10X RT Buffer in an adjusted total volume of 20 μL using molecular grade water. For each sample, a no-RT control was included to confirm the absence of contaminating DNA. Synthesis of cDNA was performed in a Mastercycler Gradient Thermocycler (Flexigene, Cambridge, UK) under the following conditions: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and a cooling step to 4°C. The cDNA was [then??] stored at −20°C.

RT-qPCR

Quantitative PCR amplification was conducted using the ABI 7300 Real Time PCR System (Applied Biosystems, Singapore) and using the GoTaq®qPCR Master Mix (Promega, Leiden, Netherlands). The primers, already used in previous studies (Pfaffl 2001; Rashid *et al.*, 2006; Medellin-Peña *et al.*, 2007; Delcenserie *et al.*, 2012), were synthesized by Eurogentec (Liège, Belgium) (Table 2). The PCR was performed in a total volume of 25 μL, containing 12.5 μL GoTaq 2X Master Mix, 1 μL of forward primer, 1 μL of reverse primer, 0.25 μL of carboxy-X-rhodamine, 2 μL of diluted cDNA and 8.25 μL of molecular grade water. The PCR conditions were as follows: denaturation program: 95°C for 10 min; amplification and quantification repeated 40 times: 95°C for 30 s, 54°C for 30 s and 72°C for 30 s; melting curve program: 60 to 95°C with a heating rate of 0.1°C s^{-1}, and finally a cooling step to 40°C. Cycle threshold (Ct) values were determined using the ABI 7300 System SDS software. Two housekeeping genes, *gst* (glutathione transferase) and *gnd* (6-phosphogluconate dehydrogenase), were tested. The transcript levels were normalized to the gene expression of *gst*, as expression of this gene was found to be the most stable under the different treatments. The relative changes in gene expression were calculated using formula (1) as described by Pfaffl (2001), where E is the efficiency of the qPCR. The experiments were replicated three times.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}(\text{control}-\text{sample})}}{(E_{\text{ref}})^{\Delta Ct_{\text{ref}}(\text{control}-\text{sample})}}. \quad (1)$$

Statistical analysis

The data are presented as means ± standard deviations of three replicates. Statistical analysis was performed using ANOVA. When necessary, a Student's *t*-test was used to assess the differences between test groups, where $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

In order to study the effects of OH and CA on virulence gene transcription, some virulence genes *ler*, *stx2B*, *fliC* and *luxS* were monitored in a strain of EHEC O157:H7. The effect on both components of exposure to subMICs was assessed using RT-qPCR. Two subMICs of OH and CA were tested: (1) 0.005% and (2) 0.008%. Results showed that these concentrations did not negatively affect bacterial growth of *E. coli* O157:H7 after 4 h incubation at 37°C. The average ODs of EHEC O157:H7 after 4 h exposure to OH and CA at 600 nm were 1.228, 1.127, 1.142 and 0.906 for OH1, OH2, CA1 and CA2, respectively. All the results were normalized to the expression of the *gst* housekeeping gene and adjusted according to the efficiency of each pair of primers (Pfaffl 2001; Tellez *et al.*, 2012). Results showing PCR efficiency, Ct values and standard deviations are presented in Table 3.

The results were analysed at the early-exponential (4 h) growth phase, as described previously (Bergholz *et al.*, 2007; Tellez *et al.*, 2012). The results demonstrated that *E. coli* O157:H7 grown in the presence of OH showed inhibition of the transcription of the four tested genes at both subMICs (Fig. 1 a). The *luxS* gene was the most affected (ratios of −64.04 and −130.11 at subMICs of 0.005% and 0.008%, respectively; $P \leq 0.05$), followed by the *ler*, *stx2B* and *fliC* genes. OH essential oil is a complex of different molecules, with CA being its major component (Table 1). Therefore, in order to further investigate the effect on EHEC O157:H7, CA was also analysed at the same subMICs. The results again showed a strong effect on the downregulation of virulence gene transcription following the same pathway observed with the parent oregano essential oil. The expression of *luxS* was the most affected (ratios of −56.64 and −142.16 for

Table 3. Effect of *O. heracleoticum* essential oil and CA on expression (Ct values ± SD) of virulence genes of EHEC O157:H7 ATCC 35150 after 4 h incubation (raw data before normalization).

Gene	PCR efficiency[d] (%)	No treatment[a]	OH1[b]	OH2[b]	CA1[c]	CA2[c]
gst	96.2	24.96 ± 1.68	26.16 ± 0.98	24.46 ± 0.26	23.99 ± 0.99	26.71 ± 0.43
gnd	99.5	24.64 ± 2.42	24.82 ± 1.24	25.80 ± 0.87	22.14 ± 1.25	27.03 ± 0.78
ler	85.7	25.18 ± 1.56	30.47 ± 1.36	31.00 ± 2.83	31.55 ± 0.89	33.88 ± 1.64
stx2B	96.4	24.35 ± 1.46	28.02 ± 0.17	28.44 ± 1.25	24.88 ± 1.01	29.63 ± 0.19
fliC	96.4	22.72 ± 1.33	25.43 ± 0.19	26.09 ± 1.30	22.41 ± 0.28	27.61 ± 0.47
luxS	95.9	21.86 ± 1.08	28.75 ± 0.61	29.50 ± 0.99	27.63 ± 0.86	30.42 ± 1.22

[a]No treatment = *E. coli* O157:H7 grown in brain–heart infusion (BHI) broth for 4 h.
[b]Treatment with oregano essential oil: OH1 (0.05 μL mL^{-1}) and OH2 (0.08 μL mL^{-1}).
[c]Treatment with CA: CA1 (0.05 μL mL^{-1}), CA2 (0.08 μL mL^{-1}).
[d]PCR efficiency $E = [(10^{(-1/\text{slope})})/2] \times 100\%$.

subMICs 0.005% and 0.008%, respectively; $P \leq 0.05$) (Fig. 1 b), followed by expression of the genes *ler*, *stx2B* and *fliC*. The modulation of virulence gene transcription with the essential oil and CA was dose dependent except in the case of the gene *stx2B*. This gene reached a maximal downregulation even in the presence of the lowest CA concentration. A lower subMIC of CA could therefore have been investigated in that case. On the other hand, the modulation of virulence gene expression was not affected by the degree of purification of the tested samples (oregano essential oil and CA). Both the essential oil and CA showed a similar level of efficacy, except in the case of the *stx2B* and *fliC* genes, where the essential oil induced the greatest downregulation.

It was interesting to observe that after 4 h exposure to treatment with OH and CA, a strong inhibition of the gene *luxS* in EHEC O157:H7 was obtained. The gene *luxS* is well known for its role in QS regulation and is involved in regulating the gene expression of some virulence factors such as *ler* and *fliC* in *E. coli* O157:H7 (Xavier and Bassler 2003; Medellin-Peña *et al.*, 2007). This may have influenced the downregulation of the other related virulence genes. In their study of virulence gene expression in *Campylobacter jejuni*, Mundi *et al.* (2013) tested a *luxS* mutant strain in parallel with the corresponding wild strain. It would be interesting to test further the effects of OH and CA on a *luxS* mutant strain of *E. coli* in order to discover whether the downregulation of the gene virulence expression is still observed.

The results of our study and the studies discussed above strongly suggest that CA may be the major active component in oregano essential oil that is able to affect virulence gene expression and also the expression of the *luxS* gene involved in QS. This hypothesis is supported by the work of Burt *et al.* (2007), who reported the inhibition of flagellin synthesis and the decrease in motility of EHEC O157:H7 with increasing subMICs of CA. It is important to note that bacterial flagella are responsible for the activation of host immune response during infection (Burt *et al.*, 2007). CA has also been reported to inhibit the motility of *Salmonella* Typhimurium and *C. jejuni* at subMICs, and to demonstrate the ability to reduce or inhibit cellular infection (Inamuco *et al.*, 2012; van Alphen *et al.*, 2012). Landau and Shapira (2012) found that adaptation of EHEC to menthol reduced the expression of *ler* and increased the expression of the gene *cpsB10*, responsible for encoding one enzyme for colanic acid production. These arguments support findings with regard to the inactivation of *ler* in EHEC O157:H7. Takemasa *et al.* (2009) reported that of 20 spices tested, allspice extract and its main component, eugenol, were able to reduce Stx production in EHEC O157:H7. They also indicated that one of the possible mechanisms for this was the binding of eugenol to conserved membrane histidine sensor kinase (QseC), which prevents toxin gene activation (Takemasa *et al.*, 2009). CA and eugenol belong to the phenolic group of compounds, which contain the functional –OH group. This information seems to be relevant to the present study as downregulation in *stx2B* gene transcription is likely to be related to downregulation in *luxS* gene transcription induced by CA and its parent oregano essential oil. Indeed, as suggested by the findings of Sperandio *et al.* (2001), *stx* genes with the presence of a λ-like Q-dependent promoter are induced by an SOS response resulting from genes involved in the SOS response, which are regulated by QS through AI-2.

Only a limited number of studies using RT-qPCR have been carried out to investigate the effect of oregano essential oil and CA in inhibiting or reducing the expression of specific virulence genes. Five plant-derived antimicrobials including CA have been shown to demonstrate the ability to downregulate the expression of most of the tested genes in EHEC O157:H7 such as *eutB*, *eutC*, *eutR*, *agaA*, *fucA*, *fucO*, *gadA*, *gadC*, *gadX*, *sdiA*, *eae* and *ler* (Baskaran and Venkitanarayanan 2014). Baskaran and Venkitanarayanan (2014) demonstrated a 2- to 3-fold downregulation of expression of the *eae* and *ler* genes, which translated into reduced adherence and invasive abilities of EHEC O157:H7. Even though the present study showed a greater downregulation of expression of the *ler* gene (at least 13-fold) than that of Baskaran and Venkitanarayanan (2014), a discrepancy can be found when these findings are compared. This may be explained by differences in culture media environment, incubation temperature, concentrations of antimicrobials and choice of housekeeping gene for normalization. However, both studies showed the same tendency of plant-derived antimicrobials to decrease virulence gene expression in EHEC O157:H7. Using RT-qPCR analysis, Amalaradjou, Narayanan and Venkitanarayanan (2011) also reported the downregulating effect of *trans*-cinnamaldehyde on different genes (*fimA*, *fimH*, *focA*, *sfaA*, *sfaS* and *papG*) of uropathogenic *E. coli* (UPEC). The authors hypothesized that this finding was correlated to the decreased ability of UPEC to attach to and invade bladder cells. Lee *et al.* (2011) showed that broccoli extracts and two polyphenolic components, quercetin and myricetin, decreased the expression of virulence-related genes (*luxS*, *pfs*, *flhD* and *eae*) in EHEC O157:H7. Results showed that the transcription of the *luxS* and *pfs* genes was most affected by quercetin, while the transcription of the *eae* gene was downregulated by myricetin (Lee *et al.*, 2011).

To the authors' knowledge, the present study is the first to demonstrate a specific effect of oregano essential oil and its main phenolic component, CA, on the expression of virulence genes involved in the upregulation of the LEE2, LEE3 and LEE4

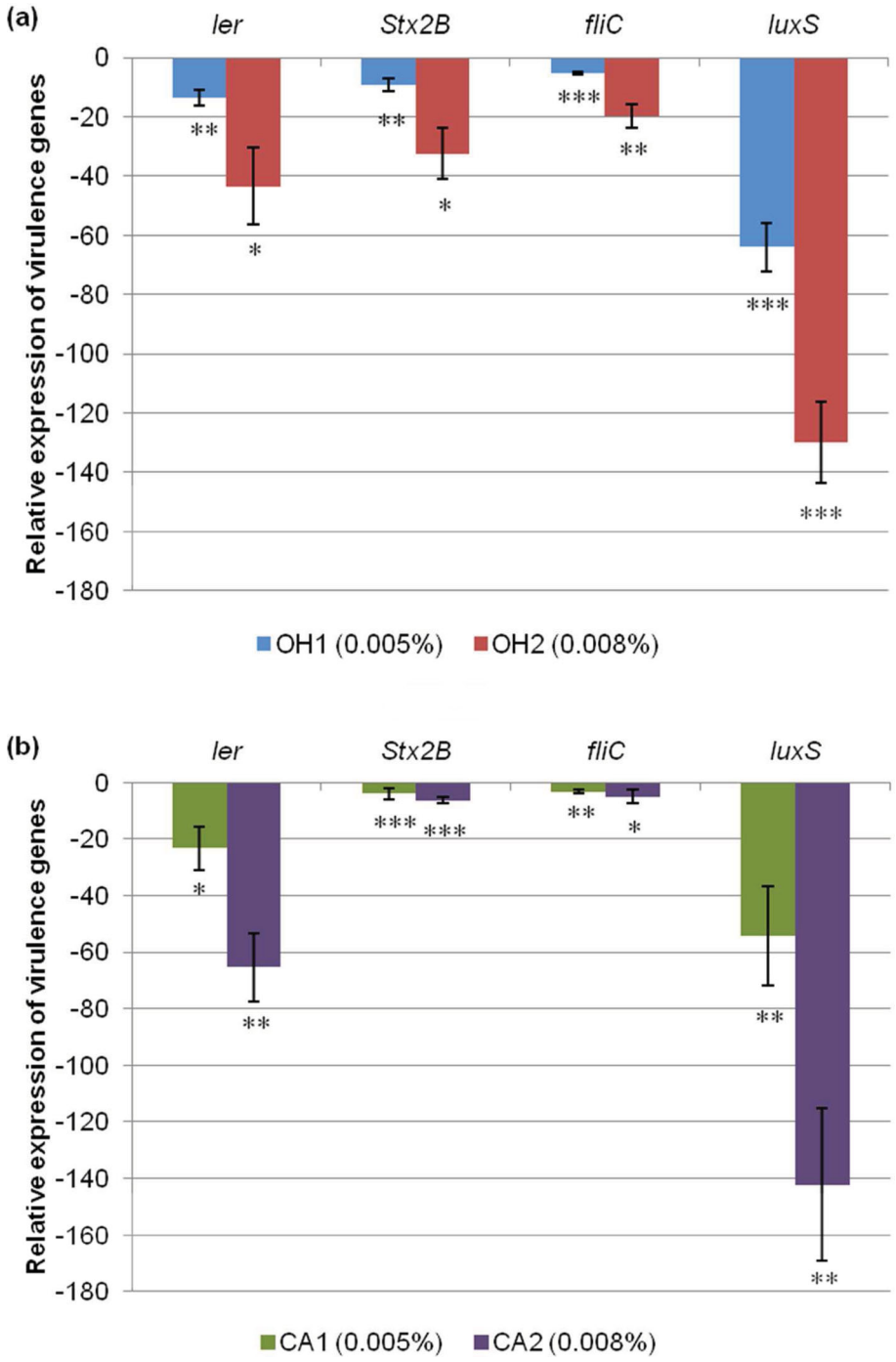

Figure 1. Effect of two sub-lethal concentrations of **(a)** *O. heracleoticum* essential oil (OH) and **(b)** carvacrol (CA) on virulence gene expression of EHEC O157:H7 after 4 h incubation. Gene expression ratios of *E. coli* O157:H7 ATCC 35150 were normalized to the expression of the regulator gene *gst*. Negative values represent downregulation of genes in the presence of essential oil or CA. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$.

promoters and of A/E lesions (*ler*), Stx production (*Stx2B*), motility (*fliC*) and in particular QS (*luxS*) for the regulation of gene expression in *E. coli* O157:H7. The results show that OH essential oil and CA exerted a significant effect by decreasing the expression of all four tested genes of *E. coli* O157:H7 at two sub-MICs (0.005% and 0.008%). The observed effect was the strongest with the *luxS* and *ler* genes. The results of this study support the potential use of essential oils as well as CA as antibacterial additives in foodstuffs. In addition, the use of very low concentrations of these oils or their main components could be advantageous in helping to lower the sensory impact when applied to foodstuffs.

CONCLUSIONS

OH essential oil and its main component CA were shown at both tested subMICs to have the ability to decrease the expression of four tested virulence genes *ler*, *stx2B*, *fliC* and *luxS* in EHEC

O157:H7. The most pronounced dose-dependent effect was observed in the gene *luxS*, which is involved in QS. The present study demonstrates a specific effect of OH essential oil and CA in downregulating the expression of virulence genes in EHEC O157:H7. These findings could represent a means for controlling the virulence of bacteria that have the potential to contaminate food. The findings also suggest that these compounds may be used as a natural alternative to antibiotics in the case of bacterial infection.

ACKNOWLEDGEMENTS

The authors acknowledge the ARES-CCD (Académie de Recherche et d'Enseignement Supérieur—Commission de la Coopération au Développement, Belgium) program for providing a scholarship to HM and also Pranarom International for providing the essential oils.

Conflict of interest statement. None declared.

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