



# Effects of Sublethal Thymol, Carvacrol, and *trans*-Cinnamaldehyde Adaptation on Virulence Properties of *Escherichia coli* O157:H7

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**ABSTRACT** Essential oils (EOs) have demonstrated wide-spectrum antimicrobial activities and have been actively studied for their application in foods as alternative natural preservatives. However, information regarding microbial adaptive responses and changes in virulence properties following sublethal EO exposure is still scarce. The present study investigated the effect of sublethal thymol (Thy), carvacrol (Car), or *trans*-cinnamaldehyde (TC) adaptation on virulence gene expression and virulence properties of *Escherichia coli* O157:H7. The results demonstrated that *E. coli* O157:H7 grown to the early stationary phase in the presence of sublethal EO showed significantly ( $P < 0.05$ ) reduced motility (reversible after stress removal), biofilm-forming ability, and efflux pump activity, with no induction of antibiotic resistance and no significant changes to its adhesion and invasion ability on a human colon adenocarcinoma (Caco-2) cell line. Reverse transcription-quantitative PCR revealed reduced expression of relevant virulence genes, including those encoding flagellar biosynthesis and function, biofilm formation regulators, multidrug efflux pumps, and type III secretion system components. This study demonstrated that Thy, Car, and TC at sublethal concentrations did not potentiate virulence in adapted *E. coli* O157:H7, which could benefit to their application in the food industry.

**IMPORTANCE** The present study was conducted to evaluate changes in virulence properties in *Escherichia coli* O157:H7 adapted to sublethal essential oils (EOs). The results demonstrated reduced motility, biofilm-forming ability, and efflux pump activities in EO-adapted *E. coli* O157:H7, with no induction of antibiotic resistance or infection (adhesion and invasion) on Caco-2 cells. Reverse transcription-quantitative PCR results revealed changes in the expression of related virulence genes. Thus, the present study provides new insights into microbial virulence behavior following EO adaptation and suggests that Thy, Car, and TC sublethal exposure did not constitute a significant risk in inducing microbial virulence.

**KEYWORDS** Caco-2 cell line, *E. coli* O157:H7, antibiotic resistance, biofilm, essential oil adaptation, motility

*Escherichia coli* O157:H7 is one of the leading causes of bacterial gastroenteritis in humans, resulting in an estimated 73,000 illness cases annually in the United States (1). Diseases caused by *E. coli* O157:H7 range from watery diarrhea to hemorrhagic colitis and life-threatening hemolytic uremic syndrome, with much higher hospitalization and fatality rates compared to other enteric pathogens such as *Salmonella* or *Campylobacter* spp. (1). According to the Centers for Disease Control and Prevention, foodborne transmission accounted for 52% of *E. coli* O157:H7 outbreaks, with contaminated beef, dairy products, ready-to-eat fresh produce, and fruits being the most commonly identified food sources (2). In addition, *E. coli* O157:H7 are able to form

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biofilm on various surfaces, which makes their elimination from food processing facilities impossible (3).

Given the importance of *E. coli* O157:H7 in human illness, it is critical that strategies controlling the risk from this pathogen are available. The increasing bacterial resistance to conventional preservatives and the consumer demand for natural food additives have encouraged research for finding novel antimicrobials. Essential oils (EOs) are gaining popularity in recent years, due to their natural origin, wide-spectrum antimicrobial activities, and generally recognized as safe (GRAS) status for food usage (4). In particular, thymol (Thy), carvacrol (Car), and *trans*-cinnamaldehyde (TC), the principal compounds of thyme, oregano, and cinnamon EOs, respectively, have been demonstrated to possess good antimicrobial activities against various foodborne pathogens, including *E. coli* O157:H7 (5), *Staphylococcus aureus* (6), *Salmonella enterica* serovar Typhimurium (7), *Pseudomonas aeruginosa* (3), and *Campylobacter jejuni* (8).

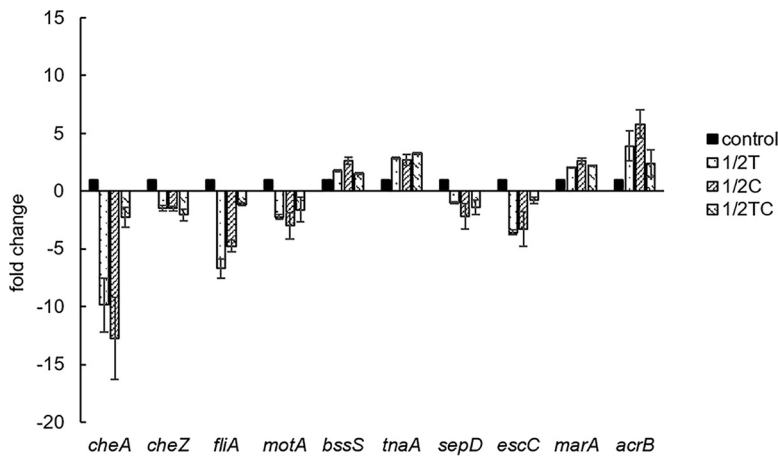
The main obstacle limiting the application of EOs in foods is that their antimicrobial dosages in food matrix are often higher than the organoleptically acceptable concentrations (9), which demands their use at sublethal concentrations in combination with other preservative methods (the hurdle approach) (10). In addition, their interaction with food components such as proteins and fats, as well as their potential loss during storage due to highly volatile nature (4), could further reduce the active concentration to below lethal level. It is well known that exposure of microorganisms to sublethal stresses such as biocides (11), weak acid, and mild heat (12) would induce microbial adaptive responses and result in increased stress tolerance and possibly higher virulence in stress-adapted bacteria (13). However, limited studies have been conducted to evaluate microbial adaptive response and virulence changes toward sublethal EO exposure.

Our previous study (14) using RNA sequencing revealed altered expression of various virulence genes in *E. coli* O157:H7 grown to early stationary phase in the presence of sublethal Thy, Car, or TC, including genes related to flagellar biosynthesis and function, chemotaxis, biofilm development, type III secretion systems (T3SS), and efflux pumps. However, the biological significance of these transcriptional changes has not yet been verified. Therefore, the present study aimed to understand the effect of sublethal EO (Thy, Car, TC) adaptation on *E. coli* O157:H7 virulence properties, including motility, biofilm formation, adherence to and invasion of the Caco-2 cell line, and antibiotic resistance. In addition, changes in the expression of related virulence genes were determined using reverse transcription-quantitative PCR (RT-qPCR).

## RESULTS AND DISCUSSION

**Changes in virulence gene expression in EO-adapted *E. coli* O157:H7.** It was observed that adaptation of *E. coli* O157:H7 to sublethal concentrations of Thy, Car, or TC resulted in the upregulation of genes encoding multidrug efflux pumps (*marA* and *acrB*) and negative regulators of biofilm formation (*tnaA* and *bssS*). On the other hand, several virulence genes related to flagellar biosynthesis and functions (*flhA* and *motA*), chemotaxis (*cheA* and *cheZ*), and T3SS (*sepD* and *escC*) were downregulated in EO-adapted bacteria, compared to nonadapted controls (Fig. 1). Similar to these results, Johnny et al. (15) reported that 30 min of incubation of *Salmonella enterica* serovar Enteritidis with sublethal TC (0.1 mg/ml) or eugenol (Eug; 0.4 mg/ml) repressed genes associated with bacterial virulence and colonization, including those conferring flagellum-associated motility, epithelial cell invasion, T3SS biosynthesis, and other surface virulence structures. It is known that exposure of *E. coli* O157:H7 to various plant-derived antimicrobials (Thy, Car, TC, and Eug) repressed genes that are critical for colonization in cattle gastrointestinal tract (16). Moreover, Amalaradjou et al. (17) revealed that sublethal TC downregulated genes that are critical for host tissue colonization in uropathogenic *E. coli*, thereby suppressing the attaching and invading ability of the bacteria.

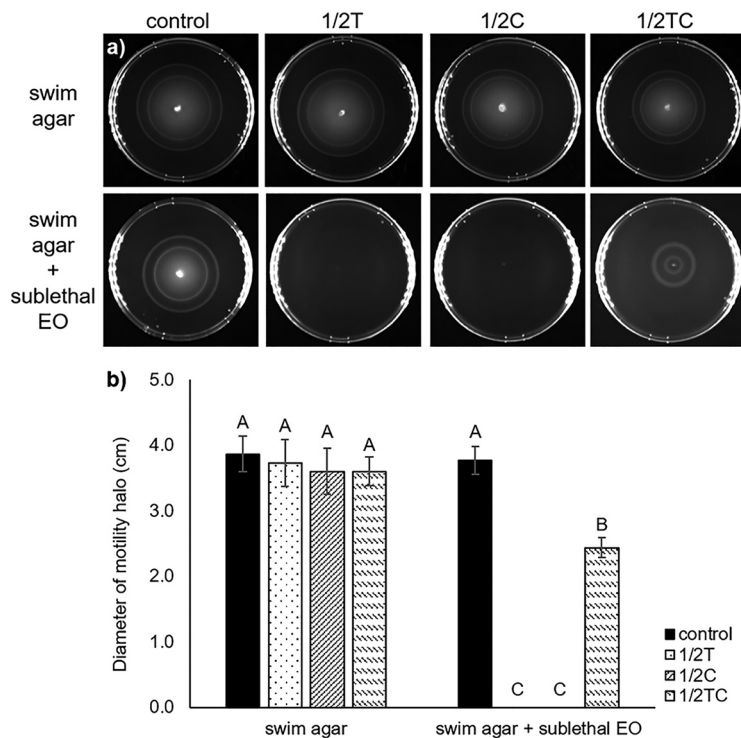
To confirm whether the observed changes in virulence gene expression would lead to observable phenotype changes, we performed experiments on EO-adapted *E. coli*



**FIG 1** Relative expression levels of virulence genes related to chemotaxis (*cheA* and *cheZ*), flagella (*fliA* and *motA*), biofilm (*bssS* and *tnaA*), the T3SS (*sepD* and *escC*), and antibiotic resistance (*marA* and *acrB*) in *E. coli* O157:H7 adapted to sublethal concentrations of thymol (1/2T), carvacrol (1/2C), or *trans*-cinnamaldehyde (1/2TC), compared to nonadapted cells (control).

O157:H7 to assess its flagellum-mediated motility, biofilm-forming ability, T3SS-dependent intestinal cell infection (adhesion and invasion), and antibiotic resistance, which are critical virulence factors for full *E. coli* O157:H7 pathogenicity.

**Changes in motility of EO-adapted *E. coli* O157:H7.** No significant difference ( $P > 0.05$ ) in the diameters of motility halos was observed between nonadapted control and EO-adapted *E. coli* O157:H7 strains, when these bacteria were incubated in swim agar for 6 h at 37°C (Fig. 2). This observation contradicted our RT-qPCR results, where



**FIG 2** Representative image (a) and diameter (b) of motility halo of *E. coli* O157:H7, nonadapted (control) or adapted to sublethal thymol (1/2T), carvacrol (1/2C), or *trans*-cinnamaldehyde (1/2TC), in swim agar without (recovery media) or with a sublethal level of EO (continuous stress media). Different capital letters (A to C) represent statistically significant ( $P < 0.05$ ) differences between control and EO-adapted cells.

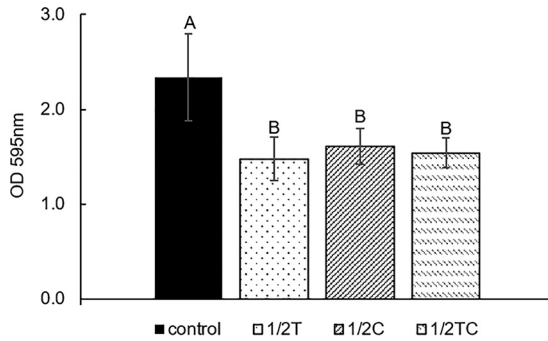
genes related to bacterial motility were found to be significantly downregulated. These include *fliA* (−1.1- to −6.7-fold), sigma factors that promote the biosynthesis of flagellar components (18); *motA* (−1.6- to −3.0-fold), components of flagellar stator complex that powers flagellar motor rotation (19); and *cheA* (−2.3- to −12.8-fold) and *cheZ* (−1.5- to −2.1-fold), components of a signaling complex that influences flagellar rotation and cell swimming behavior in response to external stimuli (20) (Fig. 1). Similar to our RT-qPCR results, Carraro et al. (21) found repression (−3.34- to −34.11-fold change) of various motility and chemotaxis genes in *E. coli* K-12 treated with polyphenols from olive mill waste. Burt et al. (5) reported that overnight incubation of *E. coli* O157:H7 with 1 mM Car inhibited flagellin synthesis, resulting in aflagellate and non-motile cells, and cell motility was negatively correlated with Car concentration.

Based on RT-qPCR results and the previously reported studies, it was hypothesized that *E. coli* O157:H7 cells might have recovered and regained the lost motility when incubated in nutrient-rich swim agar (recovery media). To confirm this hypothesis, swim agar containing different sublethal EOs (continuous stress media) was prepared and used for incubation. As expected, swim agar containing sublethal Thy or Car was found to completely inhibit microbial motility, while agar containing sublethal TC significantly ( $P < 0.05$ ) reduced motility compared to a control plate without EO (Fig. 2). This observation agrees with previously reported studies, wherein soft agar containing sublethal Car, TC, or Eug were found to significantly ( $P < 0.05$ ) reduce the motility of *S. Typhimurium* and *C. jejuni* (8, 22).

Flagellar and chemotaxis systems are essential for full pathogenesis of *E. coli*, since flagellated pathogens are able to swim toward beneficial environment and away from toxic compounds, thereby having greater survival advantages (20). Moreover, flagella play essential role in adhesion, biofilm formation, secretion of effector molecules, and the host immune system defense, which enhances pathogen virulence during infection (23). However, bacterial motility and chemotaxis systems are physiologically expensive, requiring approximately 3% of total cellular proteins to carry out these functions (24). It is known that EOs, even at sublethal concentrations, could damage microbial membranes, dissipate membrane potential, and induce intracellular ATP leakage in treated bacteria (7). Therefore, when subjected to sublethal EO stress, it is expected that *E. coli* O157:H7 would repress its motility systems and conserve energy for vital cellular activities and damage repair until a suitable growth condition was provided. Moreover, the stronger motility inhibition observed in Thy- and Car-containing plates, compared to TC-containing plates, could be explained by the stronger bactericidal activities of Thy and Car compounds (4). Thus, the present results indicate that Thy, Car, and TC present at sublethal concentrations could reduce *E. coli* O157:H7 virulence by abolishing or reducing microbial motility.

**Changes in biofilm forming ability of EO-adapted *E. coli* O157:H7.** It was observed that *E. coli* O157:H7 adapted to sublethal Thy, Car, or TC had significantly ( $P < 0.05$ ) compromised biofilm-forming ability, since it could only produce 63 to 69% of the amount of biofilm compared to that of a nonadapted control (Fig. 3). It is well documented that *E. coli* O157:H7 is able to attach, colonize, and form biofilms on various food contact surfaces and equipment, which increases the risk of food contamination during processing (25). Past studies focused mainly on using EOs as antimicrobial treatment to prevent biofilm formation (26) or destroy existing biofilms (27), whereas the results obtained in this study demonstrated that preadaptation to sublethal level of Thy, Car, or TC had an extended impact on *E. coli* O157:H7 biofilm formation, even after EO removal and nutrient replenishment.

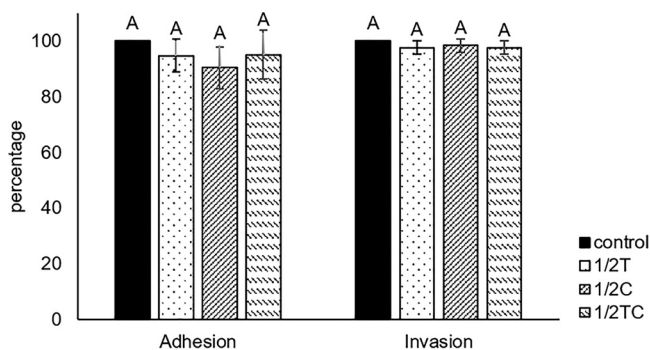
In accordance with our biofilm assay results, RT-qPCR revealed enhanced expression of the genes *tnaA* (2.7- to 3.2-fold) and *bssS* (1.5- to 2.6-fold), which were negative regulators of biofilm formation. Gene *tnaA* encodes enzyme tryptophanase, which is involved in the production of indole (28), a signaling molecule that represses biofilm formation in *E. coli* (29). Similarly, BssS was reported to reduce bacterial biofilm formation by influencing cell signaling, and the deletion of the *bssS* gene was found to



**FIG 3** Biofilm-forming ability of *E. coli* O157:H7, nonadapted (control) or adapted in 0.16 mg/ml thymol (1/2T), carvacrol (1/2C), or *trans*-cinnamaldehyde (1/2TC), in TSB. Different capital letters (A and B) represent statistically significant ( $P < 0.05$ ) differences between control and EO-adapted cells.

increase biofilm formation in *E. coli* (29). Consistent with these observations, our previous RNA sequencing study (14) revealed that multiple points of biofilm formation pathways were affected, including genes for the biosynthesis of flagella (*flhCD*, *fliAZ*, and *flgM*), poly-*N*-acetylglucosamine (*csrB*), colonic acid (*wza*), and curli (*csgD*).

**Changes in the adhesion and invasion properties of EO-adapted *E. coli* O157:H7 for the Caco-2 cell line.** Genes involved in T3SS biosynthesis and function, namely, *sepD* and *escC*, were downregulated 1.0- to 2.2-fold and 0.8- to 3.6-fold, respectively, in EO-adapted *E. coli* O157:H7 (Fig. 1). Genes *sepD* and *escC* encode essential components of T3SS, and the lack of these genes in *E. coli* mutants was found to impair the pathogen's ability to adhere and induce attaching and effacing lesions in host cells (30, 31). The RT-qPCR results prompted us to study the adhesion and invasion abilities of EO-adapted bacteria for the Caco-2 cell line, since these T3SS-dependent virulence properties were essential for full *E. coli* O157:H7 pathogenicity (32). To our surprise, no significant reduction ( $P > 0.05$ ) was found in the percentage of EO-adapted bacterial cells that attached to and/or invaded the Caco-2 cell line relative to this percentage for the nonadapted control (Fig. 4). In contrast to the present results, *S. Typhimurium* adapted to 0.08 and 0.12 mg/ml Car to log phase showed reduced invasion of, but no difference in adhesion to, human (Caco-2) and porcine (IPEC-J2) epithelial cell lines (22). Similarly, *E. coli* O157 (EDL933) and non-O157 enterohemorrhagic *E. coli* strains adapted to sublethal Car (0.016%) to stationary phase showed a 9.2 to 21.6% reduction in adherence to the human ileocecal carcinoma cell line HCT-8 (33). In contrast, van Alphen et al. (34) found that a 16-h preadaptation with sublethal Car (0.03 mg/ml) reduced *C. jejuni* invasion of INT-407 intestinal epithelial cells by 54.4% but had no effect on the invasiveness of Car-adapted *E. coli*. Therefore, it is possible that the EO-adaptive response is organism and strain dependent; more bacterial isolates should



**FIG 4** Adhesion and invasion ability of *E. coli* O157:H7, nonadapted (control) or adapted in 0.16 mg/ml thymol (1/2T), carvacrol (1/2C), or *trans*-cinnamaldehyde (1/2TC), to the Caco-2 cell line.

**TABLE 1** MICs of various antibiotics against *E. coli* O157:H7, either nonadapted (control) or adapted to sublethal thymol (1/2T), carvacrol (1/2C), or *trans*-cinnamaldehyde (1/2TC)

Antibiotic	Avg MIC ( $\mu\text{g/ml}$ )			
	Control	1/2T	1/2C	1/2TC
Tetracycline	4	4	4	4
Ampicillin	5	4	5	4
Nalidixic acid	7	6	6	6
Gentamicin	6	6	4	5
Chloramphenicol	20	18	24	16
Streptomycin	22	19	19	21
Novobiocin	630	692	692	630

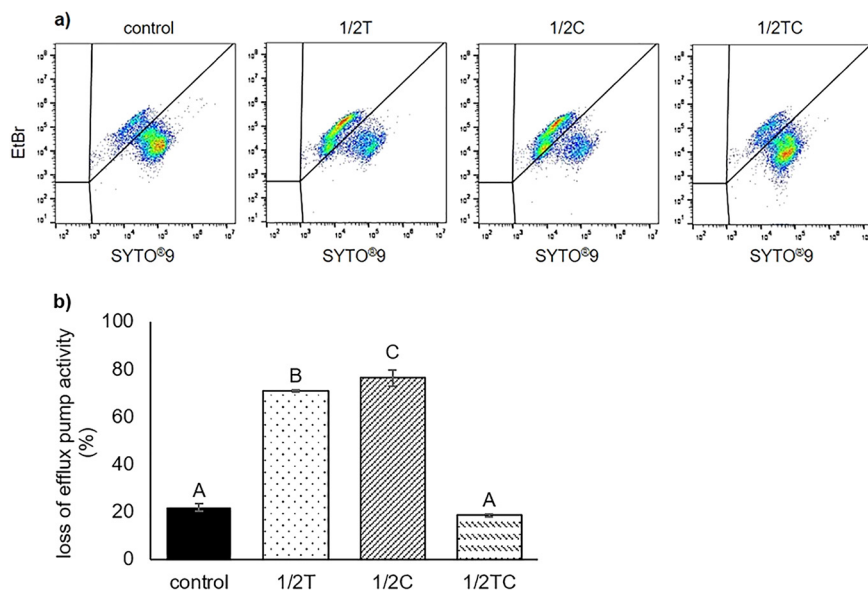
be evaluated in future studies to gain a better understanding of the microbial adaptive response to sublethal EO adaptation.

One possible reason for the discrepancies between RT-qPCR and Caco-2 assay results could be that the repression of the T3SS gene expression levels in EO-adapted bacteria was reversed during incubation with the Caco-2 cell line. Giron et al. (35) previously demonstrated that the presence of HeLa epithelial cells stimulated and restored the expression of surface adhesive structure in enteropathogenic *E. coli* strain due to signaling molecules produced by eukaryotic cells. Furthermore, since *E. coli* O157:H7 possesses a complex intestine adherence system consisting of more than 25 colonization factors (30), it is also possible that the production of other virulence factor masked the defects in T3SS and enabled normal microbial infection of the intestinal cell line.

**Changes in antibiotic resistance and efflux pump activities of EO-adapted *E. coli* O157:H7.** The effects of sublethal EO exposure on the development of *E. coli* O157:H7 antibiotic resistance were minimal when evaluated by standard MIC assessment criteria (Table 1), where an MIC difference of  $\leq 2$ -fold was not considered significant (36). In addition, both control and EO-adapted bacteria remained “susceptible” to all antibiotics evaluated according to the CLSI MIC breakpoint classification (36). On the other hand, the efflux pump genes *marA* and *acrB* were upregulated 2.1- to 2.6-fold and 2.4- to 5.8-fold, respectively, in EO-adapted bacteria compared to the nonadapted control (Fig. 1). In *E. coli*, the multiple antibiotic resistance (*mar*) gene *marA* is known to be a global regulator that controls the expression of more than 60 chromosomal genes, resulting in increased pathogen resistance to antibiotics and other environmental hazards (37). Among the MarA-regulated gene products, multidrug efflux pumps such as AcrAB can expel a wide variety of structurally unrelated antibiotics, including quinolones,  $\beta$ -lactams, tetracyclines, and aminoglycosides (38), and were found to play a major role in *E. coli* multidrug resistance (MDR) (39).

There has been a growing concern on the use of antibacterial agents in the food industry, as chronic exposure of bacteria to sublethal concentration of food preservatives (40) and disinfectants (41) has been found to increase microbial antibiotic resistance. Among the limited number of studies conducted on EO adaptation to date, inconsistent results were obtained for their induction on bacterial MDR development. Repeated exposure to EOs (tea tree EO, oregano EO, and cinnamon EO) or their major components (terpinen-4-ol, eugenol, and TC) has a minimal impact on antibiotic resistance development (MIC change  $\leq 2$ -fold) in *E. coli*, *P. aeruginosa*, and *S. aureus* (42–44). In contrast, habituation of *Salmonella* spp., *E. coli*, *S. aureus*, and methicillin-resistant *S. aureus* to tea tree EO (45) or linalool (46) was found to increase the MIC by up to 32-fold in adapted bacteria versus standard antibiotics. Our results demonstrate that although sublethal Thy, Car, and TC induced the expression of efflux pump genes, they had little impact on the development of MDR in adapted *E. coli* O157:H7 and were thus not considered a potential risk to the health of the general public.

To better understand the seemingly contradictory results from the MIC assay and RT-qPCR, we measured the loss of efflux pump activity in nonadapted and EO-adapted bacteria with SYTO9/ethidium bromide (EtBr) stain using flow cytometry. SYTO9 enters



**FIG 5** Representative flow cytometry plots (a) and calculated percentage loss of efflux pump activity (b), assessed using SYTO9/EtBr stain, in nonadapted *E. coli* O157:H7 (control) and cells adapted to sublethal thymol (1/2T), carvacrol (1/2C), or *trans*-cinnamaldehyde (1/2TC). Different capital letters (A to C) represent statistically significant ( $P < 0.05$ ) differences between control and EO-adapted cells.

both live and dead bacteria, binds to nucleic acids, and emits green fluorescence. On the other hand, EtBr enters only cells with inactive efflux pumps, displaces SYTO9 from nucleic acid, and emits red fluorescence (47). It was observed that significantly ( $P < 0.05$ ) greater amounts of *E. coli* O157:H7 lost their efflux pump activities after adaptation to sublethal Thy (70.9%  $\pm$  0.4%) or Car (76.2%  $\pm$  3.5%) compared to nonadapted controls (21.9%  $\pm$  0.1%), whereas no significant difference ( $P > 0.05$ ) was found between nonadapted controls and TC-adapted cells (18.4%  $\pm$  0.5%) (Fig. 5). Previous studies suggested that some natural products were also substrates for multidrug efflux pumps and could “block” the activities of these efflux pumps from expelling antibiotics by competitive binding and saturation of the pumps, thereby restoring the susceptibility of MDR bacteria against standard antibiotics (48, 49). Results from the present study provided a different perspective, i.e., that EOs (particularly Thy and Car) could directly disrupt efflux pump activities, which could be the consequence of EO-protein interaction or due to the depletion of cellular energy after membrane disruption (47). Therefore, the enhanced expression of efflux pump genes could be the bacteria’s attempt to balance stresses imposed by EOs, so they could continue to expel harmful intracellular compounds (e.g., metabolic by-products) and maintain a normal cellular environment conducive for bacterial growth.

**Conclusion.** The present results show that *E. coli* O157:H7 grown to the early stationary phase in the presence of sublethal Thy, Car, or TC shows altered virulence gene expression, temporary suppression of motility (reversible after stress removal and nutrient replenishment), and reduced biofilm-forming ability. No significant effect was found on the abilities of EO-adapted *E. coli* O157:H7 to adhere to and invade the Caco-2 cell line. Furthermore, EOs (especially Thy and Car) at sublethal concentrations reduced efflux pump activities but did not induce antibiotic resistance in adapted bacteria. Overall, adaptation to sublethal Thy, Car, or TC did not enhance the virulence of *E. coli* O157:H7, and thus these EOs could be explored further as natural antimicrobials or disinfectants for application in the food industry.

## MATERIALS AND METHODS

**Bacterial strain and culture conditions.** A frozen stock of *E. coli* O157:H7 (ATCC 35150) was purchased from the American Type Culture Collection (ATCC; Manassas, VA) and stored at  $-80^{\circ}\text{C}$  in Cryoinstant vials with porous beads (DeltaLab, Barcelona, Spain). Frozen culture was activated in sterile

**TABLE 2** Primers used in this study

Function	Gene	Sequence (5'–3')	Product size (bp)
Housekeeping	16S rRNA	F: AGAGGATGACCAGCCACAC	194
		R: CGGGTAACGTCAATGAGCAAAG	
Chemotaxis	<i>cheA</i>	F: AGGCTATTGCGTACCAGGTG R: AACTCGGCAAGCAGGTAGAA	109
	<i>cheZ</i>	F: ATCGTCCACCTGATCCTGAC R: AGTTGCTGATGGTGTCTTTG	137
Flagella	<i>fliA</i>	F: CTATTGCCTGTGCCACTTCA R: GCTATGACGCCCTACAAGGA	141
	<i>motA</i>	F: GGTGCGTAACCGTTCAGATT R: TTTCTCGGCATTTTATTGG	135
Biofilm	<i>bssS</i>	F: TCCTGCTCGGACTTATTTGG R: ATTCAGACTCATCCGCTCGT	104
	<i>tnaA</i>	F: CAAAGAAGCCTTCGATACGG R: GCGGAGTTACTGGTGTATGGT	135
T3SS	<i>sepD</i>	F: TGCTTTCTTGCACGATTTTG R: GGCCAGGATATTGCAGAGTC	109
	<i>escC</i>	F: GAGAAAAGGTGGCGTCTCTG R: CGGTAAGGCTATTCGAGCTG	93
Efflux pumps	<i>marA</i>	F: AATACATCCGCAGCCGTAAG R: CGGCGGAACATCAAAGTAAT	146
	<i>acrB</i>	F: ATCAATGGCGAGATTTCTG R: GCATTGCGGAGATAATGGTT	137

tryptic soy broth (TSB; Oxoid, Hampshire, England) by two consecutive transfers (37°C, 24 h), and daily transfer was performed to maintain cell viability. Cells were washed twice in 1× phosphate-buffered saline (PBS; Vivantis, Inc., Oceanside, CA) with centrifugation (5,000 × *g*, 4°C, 5 min) and diluted in TSB to an appropriate concentration before use.

**Preparation of essential oil-adapted cells.** Thy, Car, and TC were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Stock solutions of EOs were prepared in 95% ethanol at 100 mg/ml and stored at 4°C in the dark until use. To prepare EO-adapted bacteria, an overnight *E. coli* O157:H7 culture was inoculated (10<sup>7</sup> CFU/ml) into 10 ml of fresh TSB containing 1.6% (vol/vol) ethanol (nonadapted control) or 0.16 mg/ml Thy, Car, or TC, a sublethal concentration that was determined to have no inhibitory effect on bacterial growth (data not shown), followed by incubation at 37°C until the early stationary phase. In the present study, “EO-adapted bacteria” is defined as a “bacterial population that is able to grow in the presence of EO to stationary phase, with a final population of 9.0 ± 0.5 log CFU/ml, within 24 h of incubation.”

**Effect of EO adaptation on virulence gene expression.** Early-stationary-phase nonadapted and EO-adapted *E. coli* O157:H7 cultures were stabilized with RNAlater bacterial reagent (Qiagen, Hilden, Germany), and RNA extraction was performed using an RNeasy minikit (Qiagen) with on-column DNase digestion according to the manufacturer’s protocols. The quality and concentration of extracted RNA were verified using NanoDrop 1000 (Thermo Fisher Scientific, Inc., Wilmington, DE), and the integrity was checked by agarose gel electrophoresis. Template cDNA was synthesized from 1 μg of extracted total RNA by using the GoScript reverse transcription system (A5000; Promega) and used as the template for qPCR with a SYBR Select Master mix kit (catalog no. 4472953; Applied Biosystems). Each qPCR mixture (total volume, 25 μl) consisted of 1 μl of cDNA, 1 μl of each forward and reverse primer, 10 μl of SYBR green supermix, and 7 μl of nuclease-free water. The 16S rRNA gene was used as an endogenous control for normalization within samples. Forward and reverse PCR primers for the virulence genes were designed using Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>) (Table 2).

Real-time PCR was performed using a StepOnePlus real-time PCR system (Applied Biosystems), with the following thermal cycling conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Specificity of PCR was determined with melting-curve analysis (60 to 95°C with a heating rate of 0.3°C/s) and agarose gel electrophoresis. The relative changes in gene expression in EO-adapted cells compared to nonadapted control were calculated using the 2<sup>-ΔΔCT</sup> method (50).

**Effect of EO adaptation on motility.** The swimming motility of *E. coli* O157:H7 was evaluated as previously described (26). Swim agar plates were prepared by adding 25 ml of motility agar (Luria-Bertani broth containing 0.3% agar; Sigma-Aldrich) into a petri dish (94 by 16 mm; Sigma-Aldrich) and allowed to solidify overnight at room temperature. Then, 25 μl of an early-stationary-phase *E. coli* O157:H7 culture (ca. 10<sup>9</sup> CFU/ml), nonadapted or EO adapted, was inoculated into the center of swim agar plate. The diameter of each motility halo (bacterial migration distance from the site of inoculation) was measured after 6 h of incubation at 37°C.

**Effect of EO adaptation on biofilm formation.** Early-stationary-phase nonadapted and EO-adapted *E. coli* O157:H7 cells were diluted to a final concentration of 10<sup>5</sup> CFU/ml in fresh TSB, and 200 μl of the



diluted culture was placed in a sterile 96-well plate (Thermo Fisher Scientific). After 24 h of incubation at 37°C, the amount of biofilm formed was quantified using a crystal violet staining method according to Lee et al. (51). Briefly, each well was washed three times with sterile deionized (DI) water to remove loosely attached cells and then stained with 200  $\mu$ l of 0.1% (wt/vol) crystal violet solution for 20 min at room temperature. Subsequently, the staining solution was discarded, rinsed three times with DI water, and incubated with 200  $\mu$ l of 95% ethanol at 4°C for 15 min to extract dye from biofilm. Absorption was then measured at 595 nm using a Multiskan FC microplate photometer (Thermo Fisher Scientific).

**Effect of EO adaptation on the ability to adhere to and invade the Caco-2 cell line.** The Caco-2 human colon adenocarcinoma cells (ATCC HTB-37), purchased from the ATCC, were maintained in Dulbecco modified Eagle medium (Invitrogen/Life Technologies, Carlsbad, CA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Invitrogen) at 37°C in 5% CO<sub>2</sub>. The growth medium was changed every 3 days, and cells were passaged once a week. For infection experiments, Caco-2 cells were seeded into 12-well cell culture plate (Sigma-Aldrich) at 10<sup>5</sup> cells per well and grown to semiconfluence before use.

Bacterial infection assay of Caco-2 cell line was performed according to Inamuco et al. (22). For adhesion assay, semiconfluence Caco-2 monolayers were washed three times with 1× PBS, and each well was infected with 1 ml 10<sup>6</sup> CFU/ml nonadapted or EO-adapted *E. coli* O157:H7 (multiplicity of infection = 10). After 2 h of incubation at 37°C in 5% CO<sub>2</sub>, the infected monolayers were washed three times with 1× PBS and lysed in 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich) for 15 min at room temperature. The resulting suspensions were plated onto tryptic soy agar (TSA; Oxoid) with appropriate dilutions, and the number of adhesion bacteria was counted after 24 h of incubation at 37°C. For invasion assay, the infected Caco-2 monolayers were washed three times with 1× PBS and then incubated with 1 ml of 250  $\mu$ g/ml gentamicin (Sigma-Aldrich) at 37°C in 5% CO<sub>2</sub> for 2 h to kill extracellular bacteria. The treated Caco-2 monolayers were then washed and lysed, and the number of invasion bacteria was counted by TSA plating, as described above. The percentages of adhesion and invasion bacteria were calculated by dividing the number of adhered or invasion cell over inoculum, and final results are expressed as percentages relative to that of control (control = 100%).

**Effect of EO adaptation on antibiotic resistance.** Antibiotic resistance of nonadapted and EO-adapted *E. coli* O157:H7 was evaluated using the Clinical and Laboratory Standards Institute (CLSI) microdilution method (36). Briefly, a 2-fold serial dilution of each antibiotic was prepared in a sterile 96-well plate, and equal volumes (100  $\mu$ l) of antibiotic and bacterial solution (final inoculum level, 10<sup>5</sup> CFU/ml) were mixed. After 24 h of incubation at 37°C, 10  $\mu$ l of 0.01% (wt/vol) resazurin (Sigma-Aldrich) solution was added to each well, followed by incubation for 2 h at 37°C before visual examination of the color change. The MIC in this study was defined as “the minimum EO concentration that inhibited microbial growth, as determined by a lack of color change in resazurin indicator.”

**Effect of EO adaptation on efflux pump activity.** To validate the effect of sublethal EO adaptation on *E. coli* O157:H7 efflux pump activity, flow cytometry analysis was performed using SYTO9 (Molecular Probes; Thermo Fisher Scientific) and EtBr (Sigma-Aldrich) (52). Briefly, nonadapted and EO-adapted *E. coli* O157:H7 cell cultures were diluted in 0.2- $\mu$ m-pore-size-filtered PBS to 10<sup>6</sup> CFU/ml, and a 200- $\mu$ l aliquot was withdrawn and stained with SYTO9/EtBr mixed probes at working concentrations of 5 and 30  $\mu$ M, respectively. After incubation for 15 min at room temperature, measurements were made using a BD Accuri C6 flow cytometry device (Accuri Cytometers, Inc., Ann Arbor, MI). Signals for SYTO9 and EtBr were collected at the FL1 and FL3 detectors, respectively.

**Statistical analysis.** Experiments were performed in duplicate and repeated for at least three times ( $n = 6$ ). Results are presented as averages  $\pm$  the standard deviations, and statistically significance differences ( $P < 0.05$ ) were determined by one-way analysis of variance with Tukey *post hoc* test, using the IBM SPSS statistical software (v20; SPSS, Inc./IBM Corporation, Armonk, NY).

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