Inactivation of *Listeria monocytogenes* and *Escherichia coli* O157:H7 Biofilms by Micelle-Encapsulated Eugenol and Carvacrol

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**ABSTRACT**

Carvacrol and eugenol were encapsulated in micellar nonionic surfactant solutions to increase active component concentrations in the aqueous phase and used to treat two strains of *Listeria monocytogenes* (Scott A and 101) and two strains of *Escherichia coli* O157:H7 (4388 and 43895) grown as biofilms in a Centers for Disease Control and Prevention reactor. *L. monocytogenes* biofilms were grown in two different growth media, 1:20 TSB and Modified Welchimer’s broth (MWB), while *E. coli* O157:H7 was grown in M9. In general, *L. monocytogenes* strains were more resistant to both micelle-encapsulated antimicrobials than *E. coli* O157:H7 strains. The two antimicrobials were equally effective against both strains of *E. coli* O157:H7, decreasing viable counts by 3.5 to 4.8 log CFU/cm² within 20 min. For both bacteria, most of the bactericidal activity took place in the first 10 min of antimicrobial exposure. Biofilm morphology and viability were assessed by the BacLight RedoxSensor CTC Vitality kit and confocal scanning laser microscopy, revealing an increasing number of dead cells when biofilms were treated with sufficiently high concentrations of carvacrol- or eugenol-loaded micelles. This study demonstrates the effectiveness of the application of surfactant-encapsulated essential oil components on two pathogen biofilm formers such as *E. coli* O157:H7 and *L. monocytogenes* grown on stainless steel coupons.

*Listeria monocytogenes* and *Escherichia coli* O157:H7 are pathogens that have been associated with numerous outbreaks of foodborne diseases from a variety of foods such as undercooked ground beef, cheese, and vegetables (11, 32, 47). *L. monocytogenes* (6, 8, 12, 13, 32) and *E. coli* O157:H7 (7, 38, 40, 43) may attach and form biofilms on stainless steel surfaces, the most common material used in food processing plants. Biofilms have been defined as matrix-enclosed microorganisms that adhere to a surface and/or to each other, producing a dynamic environment in which the component microbial cells may reach homeostasis, and may optimally organize to make use of all available nutrients (29). Even at low levels of cell numbers, pathogenic biofilms can become hazardous sources of contamination for foods that touch or pass over contaminated surfaces (28). From a public health perspective, biofilm contamination with *L. monocytogenes* and/or *E. coli* O157:H7 is of particular concern to food processors, since both bacteria have exhibited higher resistance to sanitizers and antibacterial agents when grown as biofilms or attached cells instead of as planktonic cultures (5, 12, 42, 43). An increasing number of studies report that these pathogenic biofilms are able to survive exposure to chemical sanitizers such as alkaline, acid, or neutral detergents and chlorine (17, 22). For example, pathogenic biofilms have been found to survive enzyme foam–based cleaning agent treatments in floor drains of poultry processing facilities (47).

Eugenol derived from clove oil and carvacrol found in oregano and thyme oils are two essential oil components that exhibit antimicrobial activity against foodborne pathogens (25, 31). For example, a reduction of bacterial counts of 2 to 3 log CFU/ml upon application of carvacrol has been observed (27). To date, the majority of reported research with eugenol and carvacrol has focused on studying the antimicrobial activity of these compounds against planktonic cultures (3, 4, 9, 23), although their low water solubility (<0.1 mg/ml) makes the two essential oil compounds difficult to use for disinfectant purposes (9). We previously demonstrated that encapsulating eugenol and carvacrol in surfactant micelles inhibits the growth of *L. monocytogenes* and *E. coli* O157:H7 both in planktonic cultures (15) and in colony biofilms, i.e., bacteria grown on a semipermeable membrane placed on an agar plate (35). In the latter study, a reduction of bacterial counts of >4 log was observed (35). The colony biofilm model is considered a good screening tool to evaluate the effectiveness of a high number of bactericide compounds, but it does not represent well the formation process of mature biofilms found in the food industry.

In the present work, the antimicrobial activity of carvacrol and eugenol encapsulated in Surlynol 485W
micelles against 48-h-old *E. coli* O157:H7 and *L. monocytogenes* biofilms grown on stainless steel surfaces in a Centers for Disease Control and Prevention (CDC) biofilm reactor was evaluated. The efficacy of the antimicrobial delivery system was assessed by CFU enumeration of the viable cells and by visualization of viable cells, using a BacLight RedoxSensor CTC Vitality kit and confocal scanning laser microscopy in both control and treated biofilms.

**MATERIALS AND METHODS**

**Preparation of antimicrobial-containing Surlynol 485W micelles.** Surfactant micelles containing eugenol and carvacrol were prepared by dispersing the nonionic surfactant Surlynol 485W (Air Products and Chemical, Inc., Allentown, PA), approved for food contact surfaces, in double-distilled and deionized water at room temperature to obtain a surfactant stock solution with a surfactant concentration of 5% (wt/wt) (15, 16). Eugenol (4-allyl-2-methoxyphenol) and carvacrol (5-isopropyl-2-methyl-phenol), purchased from Sigma (St. Louis, MO), were added under stirring to the surfactant solution to create micellar stock solutions containing 0.9 and 0.7% (wt/wt) eugenol and carvacrol, respectively. To ensure complete encapsulation of antimicrobials in surfactant micelles, samples were withdrawn at regular intervals and their UV-visible absorption spectra were measured. Solutions became transparent, and adsorption at 300 to 800 nm decreased to zero, indicating completion of the encapsulation process and formation of so-called microemulsions or swollen micelles (15, 16). Microemulsions were then filter sterilized by using a 0.22-um-pore-size cellulose acetate membrane (Corning, Corning, NY) and stored at 4 to 6°C until used.

**Bacterial strains and growth conditions.** Two strains of *L. monocytogenes* strains (strain Scott A, serotype 4b, and strain 101, serotype 4, Department of Food Science, University of Tennessee Culture Collection) and two strains of *E. coli* O157:H7 (ATCC 4388 and 43895) were used. Stock cultures were stored at −70°C. Working cultures were maintained on slants and were inoculated from frozen stocks monthly and stored at 4°C. For each experiment, a loopful of the culture was transferred to tryptic soy broth (TSB) or TSB with 0.6% yeast extract (TSBYE) (Difco, BD, Sparks, MD) for *E. coli* O157:H7 and *L. monocytogenes*, respectively, and incubated at 32°C for 24 h (8). Bacteria were then subcultured in either TSB or TSBYE for 18 h prior to exposure assays.

**Preparation of CDC reactor biofilms.** Biofilms were grown in a CDC biofilm reactor (Biosurface Technologies Corp., Bozeman, MT) (20). The reactor consisted of a 1-liter glass vessel (type 304, diameter of 1.27 cm and thickness of 3.0 mm) that served as biofilm growth surfaces. The chips were cleaned before each experiment according to the manufacturer’s instructions. The vessel was equipped with a baffled magnetic stir bar to provide a constant flow of 80 rpm in conjunction with a stir plate. After the reactor system was assembled, the vessel was autoclaved, filled with 350 ml of filter-sterilized Modified Welshimer’s broth (MWB) or 1:20 TSB for *L. monocytogenes* and M9 media (Difco) for *E. coli* O157:H7, and autoclaved for 15 min (37). A suspension of the planktonic culture was inoculated at 1% into the vessel. The CDC biofilm reactor was operated in batch for the first 24 h at 32°C to allow for adhesion of cells. After the first 24 h, fresh medium was continuously pumped into the reactor at a flow rate of 0.77 ml/min for another 24 h at 32°C using a Master Flex peristaltic pump (model 7518-00, Cole-Parmer Instrument Co., Vernon Hills, IL).

**Antimicrobial exposure.** Stainless steel chips with mature biofilms were removed from the reactor, washed with 10 ml of phosphate-buffered saline (PBS) and submerged into 5 ml of sterile distilled water (control), Surlynol (5%, wt/vol), or Surlynol-encapsulated eugenol (0.9%, wt/vol) or carvacrol (0.7%, wt/vol) solutions for 2, 10, or 20 min. For every treatment, a total of three chips were used. Treatments were randomized across chips that were obtained from two separate reactors. Each reactor contained 24 chips, and control chips were sampled from each reactor. Treated and control chips were washed by being agitated in 10 ml of fresh PBS to remove residual antimicrobial agents and not fully attached bacteria. The washing procedure was repeated three times, and the chips were then placed into a beaker containing 10 ml of PBS. Biofilms were removed from both sides of the chips by thorough scraping with a sterile Teflon applicator stick for about 1 min. The scraped biofilm solution was shaken with 20 to 30 (2-mm-diameter) sterile glass beads at 250 rpm for 15 min in a Gyrotory shaker, model G2 (New Brunswick, NJ), to disrupt any cell clumps. The disaggregated biofilm was then serially diluted in PBS prior to enumeration.

**Bacterial enumeration.** Serially diluted samples were plated onto TSBYE agar for *L. monocytogenes* or R2A agar (BD, Sparks, MD) for *E. coli* O157:H7, using an automated spiral plater (Spiral Biotech, Norwood, MA). The plates were incubated at 32°C for 24 h, and the viable cell numbers were counted. R2A medium is a low-nutrient medium used in environmental microbiology and was developed to recover organisms under stressed conditions (36). It contains agents (soluble starch and sodium pyruvate) to stimulate the growth of stressed and chlorine-tolerant bacteria (10, 46). It was used in this project because the colony size of *E. coli* O157:H7 was smaller (1 to 2 mm) on R2A than on high-nutrient media (2.5 to 3 mm), which allowed for more accurate counts of this organism by the spiral plater and automated counter. After incubation, the plates were read by an automated counter (Q-Count, Spiral Biotech). Three replicates of chips were used, and CFU were plotted as means with standard deviations. The minimum detectable level by the Q-Count automated counter was 510 CFU per chip or 2.3 log CFU/cm².

**Confocal laser scanning microscopy.** After treatment, the biofilm chips were stained with the BacLight RedoxSensor CTC Vitality kit (Invitrogen, Carlsbad, CA), which differentiates live from dead cells by staining living bacteria fluorescent red and all bacteria fluorescent green. While dead cells are not specifically stained, their number can be determined by subtraction of red channels from green channels. This stain was used because preliminary studies using traditional BacLight with propidium iodide (data not shown) showed us high levels of red background in our untreated biofilms of *L. monocytogenes*; this was also observed, but to a lesser degree, in biofilms of *E. coli*. This may have been due to the presence of DNA in the extracellular matrix, as was observed for *Pseudomonas aeruginosa* (46) and was recently shown in *L. monocytogenes* (21). Chips were covered with 5 mM CTC solution (1 ml; Molecular Probes Invitrogen, Eugene, OR) and incubated at 37°C in the dark for 2 h. Chips were then counterstained with 1 mM SYTO 24 (1 ml; Molecular Probes...
TABLE 1. Sensitivity of E. coli O157:H7 (ATCC 4388 and 43895) and L. monocytogenes (Scott A and 101) to eugenol and carvacrol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacterial strain</th>
<th>Media</th>
<th>Log N₀ Untreated</th>
<th>Log reduction (ΔLog N)</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>2</td>
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<tr>
<td>E. coli O157:H7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empty micelles</td>
<td>ATCC 4388</td>
<td>M9</td>
<td>7.8 ± 0.1</td>
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<td></td>
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<td>7.3 ± 0.2</td>
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<tr>
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<td>7.8 ± 0.1</td>
<td>2.9 ± 0.1</td>
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<td>M9</td>
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<tr>
<td>Carvacrol</td>
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<td>7.8 ± 0.1</td>
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<td></td>
<td>ATCC 43895</td>
<td>M9</td>
<td>7.3 ± 0.2</td>
<td>2.9 ± 0.1</td>
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<tr>
<td>L. monocytogenes</td>
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<tr>
<td>Empty micelles</td>
<td>Scott A</td>
<td>1:20 TSB</td>
<td>6.8 ± 0.1</td>
<td>0.3 ± 0.1</td>
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<td>6.5 ± 0.3</td>
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<td></td>
<td>MWB</td>
<td>7.8 ± 0.2</td>
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<td>MWB</td>
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a Biofilms (48 h) were treated with empty Surlyn 485W micelles and Surlyn 485 micelles containing 0.9% eugenol and 0.7% carvacrol for 2, 10, and 20 min. N₀ (mean of three values ± SD), initial number of cells in untreated biofilms; ΔLog N (mean of three values ± SD), difference between the cell number (Log CFU per square centimeter) of the respective control biofilm that was not treated with antimicrobials or micelles (i.e., washed with PBS only) and the cell number (Log CFU per square centimeter) of the treated biofilm. For treated samples, values followed by the same letter are not significantly different (P > 0.05).

b Cell numbers were below detectable level (2.3 log CFU/cm²).

Invitrogen) at room temperature for 15 min and then washed with PBS buffer. Stained chips were placed on a glass slide and allowed to briefly dry, and then a drop of mounting oil was added to the chip surface and covered with a cover glass. Stained biofilms were visualized by confocal microscopy. Confocal images were collected by a Nikon Eclipse C1 confocal scanning laser microscope (Nikon Instruments Inc. USA, Melville, NY) at an excitation wavelength of 488 nm and at detection wavelengths set at 605 and 515 nm for the CTC stain and SYTO 24 stain, respectively. Either single images or stacks of about 15 to 20 images across a z-depth of 50 μm, depending of the thickness of the biofilm, were collected by using a 60× objective lens. In each sample, at least three representative sites were chosen for visualization. Images were subsequently processed by the NIS Elements software for Windows (Nikon Instruments Inc. USA).

Statistics. Results of bacterial enumerations were statistically analyzed. In all cases, comparisons of the means were performed by Tukey’s test with a two-factorial design using the GLM procedure. P values of <0.05 were defined as being statistically different. All calculations were performed with SAS 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Treatment of E. coli O157:H7 biofilms grown in M9. The growth of E. coli O157:H7 (strains 4388 and 43895) in M9 during 48 h is shown in Table 1. Average control biofilms of 7.8 log CFU/cm² for strain 4388 and 7.3 CFU/cm² for strain 43895 were produced. Biofilms treated in solutions containing unloaded (empty) 5% Surlyn micelles had slightly lower cellular levels than control biofilms (0.2 to 0.5 log CFU/cm²), which was also observed with water-only controls (results not shown), suggesting that for either of the tested strains empty micelles in themselves were not antimicrobially active. Exposure to 0.9% eugenol– or 0.7% carvacrol–loaded micelles for 2 min reduced the biofilms of both E. coli O157:H7 strains significantly, by 2.9 and 2.3 log (strain 4388) and by 3.0 and 2.9 log (strain 43895), respectively. Upon further exposure (10 and 20 min), an additional cellular destruction was observed for both antimicrobial systems (Table 1). Both strains were thus sensitive to the two encapsulated antimicrobials, and the effectiveness of the encapsulated antimicrobials increased with increasing exposure time.

Treatment of L. monocytogenes biofilms grown in 1:20 TSB. L. monocytogenes grown in 1:20 TSB produced a control biofilm with averages of 6.8 and 6.5 log CFU/cm² for strains Scott A and 101, respectively (Table 1). Unloaded nonionic surfactant micelles (5%) decreased the biofilm cell numbers by <0.5 log compared with the control biofilms, except when L. monocytogenes strain Scott A was exposed for 10 min, suggesting that prolonged exposure to Surlyn may have a cell-detaching effect. L. monocytogenes strain Scott A was more sensitive to eugenol than to...
carvacrol after 2 min of exposure, as eugenol led to a 3.3-log CFU/cm² reduction compared with the 1.9-log CFU/cm² reduction achieved by carvacrol. When *L. monocytogenes* strain Scott A was exposed to 0.7% eugenol–loaded micelles for 10 and 20 min, viable cells were below detectable levels. A similar reduction (3.2 to 3.4 log CFU/cm²) was found when carvacrol-loaded micelles were used. On the other hand, *L. monocytogenes* strain 101 was more sensitive to carvacrol and a 2.7-log CFU/cm² reduction was detected after 2 min of exposure to the antimicrobial-loaded micelles. For both antimicrobials, a longer exposure time (10 and 20 min) further increased the bacterial log reduction. For example, an additional destruction of 1.2 and 1.5 log CFU/cm² in *L. monocytogenes* strain Scott A was observed after 10 and 20 min of treatment with carvacrol, respectively, but differences disappeared after a 10-min contact time.

Treatment of *L. monocytogenes* biofilms grown in MWB. *L. monocytogenes* had average control biofilm growths of 7.5 and 7.8 log CFU/cm² for strains Scott A and 101, respectively, when grown in MWB (Table 1). When grown under defined nutrient conditions, both strains were more sensitive to eugenol than to carvacrol (*L. monocytogenes* strain Scott A only after 2 min of exposure). A longer treatment (10 and 20 min) with either antimicrobial equally decreased cell numbers of *L. monocytogenes* strain Scott A by at least 3.4 and 4.3 log CFU/cm², respectively. By contrast, *L. monocytogenes* strain 101 was more resistant to the antimicrobials used, especially to carvacrol, since 20 min of exposure led to a reduction of <2 log CFU/cm². In the case of *L. monocytogenes* strain Scott A treated with 0.7% carvacrol and strain 101 treated with 0.9% eugenol, a further exposure (10 and 20 min) led to an additional cellular destruction of >2 log compared with that obtained with the 2-min treatment. *L. monocytogenes* strain 101 was more resistant to eugenol than *L. monocytogenes* strain Scott A after 2 min of exposure to the antimicrobial.

Confocal scanning laser microscopy visualization of *E. coli* O157:H7 and *L. monocytogenes* biofilms treated with essential oil compound–loaded surfactant micelles. Figure 1 shows confocal scanning laser microscopy images of treated and untreated *E. coli* O157:H7 (ATCC 4388) biofilms. In the images, all cells are stained green, and actively respiring cells, which are stained red, in the combined dual-stained cells appear yellow-orange. Confocal scanning laser microscopy images for treated and untreated *L. monocytogenes* biofilms are not shown but were similar to the images shown in Figure 1. Application of carvacrol-loaded or eugenol-loaded micelles for 2 min killed high levels of cells within the biofilm. While the control biofilm was predominantly formed by actively respiring cells, only a few cells were respiring (stained red) in the biofilm treated with carvacrol- or eugenol-loaded micelles for 2 min.

**DISCUSSION**

The CDC biofilm reactor was selected as the method for biofilm production for a number of reasons. The foremost is that this reactor system has been approved by the ASTM International Society as a standard method of biofilm growth with the organism *P. aeruginosa* (1). In addition, other researchers have observed high levels of sanitizer resistance (as indicated by the lowest log reduction) for CDC reactor–grown biofilms of both *P. aeruginosa* and *Staphylococcus aureus* when compared with a variety of other biofilm production systems (2). Thus, the CDC reactor is a well-characterized standard method that produces biofilms that show high sanitizer resistance, making this system ideal for evaluating micellar delivery systems.

Compared with other biofilm reactors, the CDC biofilm reactor has a large number of growth surfaces (24 chips) that can be treated individually, allowing for evaluation of multiple parameters in each experiment; however, the results presented in this study compare multiple reactor runs and bring into question the reactor-to-reactor variability. To address this issue, we calculated the repeatability standard deviation (SD) (34) of biofilm cell numbers of *L. monocytogenes* grown on stainless steel and found that our repeatability SD was 0.26 (results not shown). The repeatability SD calculated by others have ranged from 0.11 to 0.39 (2), which was considered to be good repeatability in comparison to other biofilm growth methods. Thus, we believe our reactor-to-reactor variability is similar to other published results and within the acceptable standards for biofilm disinfectant efficacy testing.

M9 and MWB are defined minimal media for *E. coli* O157:H7 and *L. monocytogenes*. These media better supported formation of biofilms than did diluted TSB (1:20) during incubation in the CDC reactor for 48 h (data not shown). Both *E. coli* O157:H7 (7) and *L. monocytogenes* (26) were previously found to produce greater cell density in biofilms in minimal or diluted rich media than in TSB. The two strains of *E. coli* O157:H7 (4388 and 4389S) chosen for this study produced biofilms of 7.8 and 7.3 log CFU/cm² in M9 media after 48 h of incubation (Table 1). Conversely, *L. monocytogenes* strains (Scott A and 101) produced biofilms almost 1 log smaller (6.5 to 6.8 log CFU/cm²) when grown in 1:20 TSB than when *L. monocytogenes* strains were grown in MWB (biofilm of 7.5 to 7.8 log CFU/cm²). Djordjevic et al. (8) and Moltz and Martin (32) also reported that most of the *L. monocytogenes* strains tested produced a greater biofilm in MWB than in TSBYE.

Eugenol and carvacrol in micelles at concentrations of 0.9 and 0.7%, respectively, reduced (either killed or detached) most of the bacteria within the first 2 min of exposure. Furthermore, in terms of cell reductions, *E. coli* O157:H7 was more sensitive than *L. monocytogenes* (particularly strain 101) to the action of the antimicrobials. Although contradictory susceptibilities have been reported between gram-positive and gram-negative organisms exposed to the action of a variety of essential oils (9), we have previously observed similar results in both planktonic cultures (15) and colony biofilms (34). Recently, Gill and Holley (18, 19) also found that *E. coli* O157:H7 was more sensitive than *L. monocytogenes* and *Lactobacillus sakei* to...
FIGURE 1. Representative dual-channel z-stack optical sections showing the differential fluorescence emission resulting from CTC staining of 48-h E. coli O157:H7 (strain ATCC 4388) biofilms. Corresponding fluorescent images were detected in the green (all cells) and red (dead cells) channels for (A) control biofilms, (B) biofilms treated with (empty) 5% Surfynol 485W micelles for 2 min, (C) biofilms treated with 0.7% carvacrol-loaded micelles for 2 min, and (D) biofilms treated with 0.9% eugenol-loaded micelles for 2 min. The scale bar length is 10 μm.
eugenol and carvacrol. The researchers reported that its greater hydrophobicity made E. coli more sensitive to the antimicrobials, facilitating their interaction with the bacterial membrane. While in our study the antimicrobials were delivered encapsulated in nonionic surfactant micelles, the reasoning given by Gill and Holley may nevertheless explain our findings. The inclusion of antimicrobials in a surfactant micelle resulted in 10-nm particles with micellar surfaces being composed of polar surfactant headgroups (14). Although the micelles themselves are hydrophilic since they are covered by polar headgroups, the surfactant monomers of which micelles are composed are amphiphilic and have a tendency to associate with constituents in the bacterial membranes. This process is thermodynamically driven and is more or less strong depending on the hydrophilicity of the membranes.

Gram-negative microorganisms have in addition to the cytoplasmic membrane an outer membrane made of phospholipids and lipopolysaccharides. Despite this additional protection, carvacrol has been shown to be able to disintegrate this outer membrane, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane (3). Due to the nonionic nature of the micelle, no electrostatic interactions (attractive or repulsive) between the micelles and the extracellular polymeric substances of the biofilm or the cytoplasmic membrane of the cells within the biofilm can occur. Rather, the concentration difference between nonionic micelles in the aqueous phase and the biofilm matrix likely drive their penetration inside the biofilm, thereby delivering hydrophobic antimicrobials that are usually insoluble in water (35).

The effectiveness of essential oil components as antimicrobials against both spoilage and pathogen microorganisms and fungi has led several researchers to focus their efforts on clarifying the mode of action of these compounds. For example, the action of carvacrol has been extensively studied, especially against Bacillus cereus (44). It is well known that carvacrol destabilizes the cytoplasmic membrane of microorganisms (44, 45). Carvacrol acts as a proton exchanger and reduces the pH gradient across the cytoplasmic membrane. This action may be attributed to the presence of a hydroxyl group and a system of delocalized electrons. The lack of a proton motive force along with the depletion of the ATP pool will cause the cell to eventually die (44). A recent study showed that eugenol and carvacrol caused the membranes of E. coli O157:H7 and L. monocytogenes to disrupt, thereby increasing extracellular and decreasing intracellular ATP concentrations (18). In another study, the same researchers reported that eugenol and carvacrol were capable of inhibiting the membrane bound ATPase activity of E. coli O157:H7 and L. monocytogenes (19), which includes enzymes involved in ATP generation and cellular pH regulation (39). The authors reported that this was a secondary rather than a primary cause of cell death (19).

The type and morphology of biofilms formed by L. monocytogenes vary depending on the strain and growth media. Although Kalmokoff et al. (24) observed that L. monocytogenes strains (including strain Scott A) were poor biofilm formers and adhered as single cells, subsequent studies have shown that L. monocytogenes strain Scott A forms dense aggregates of cells held together by a meshlike webbing (26) or a well-developed biofilm (12, 13) on stainless steel. In our study, both strains of L. monocytogenes grew as small microcolonies in biofilms (data not shown) when grown in either 1:20 TSB or MWB. On the other hand, the morphology of E. coli biofilms has not been as well studied as that of L. monocytogenes. Recently, Maeyama et al. (29) evaluated E. coli biofilm formation on modified Luria-Bertani medium by using confocal scanning laser microscopy. They showed that the biofilm evolved from adhered cells without aggregate formation after 3 h of incubation to a thick biofilm that covered almost the entire surface area at 24 h of incubation. We observed that E. coli control biofilms covered the majority of the coupon surface after 48 h of incubation. The use of antimicrobials clearly increased nonrespiring cells (green or “not red”) (Fig. 1).

The system composed of encapsulated hydrophobic antimicrobials in nonionic surfactant micelles has been proven to be thermodynamically and pH stable (15). Furthermore, it is effective against both colony biofilms (35) and 48-h-old mature biofilms of E. coli O157:H7 and L. monocytogenes as has been shown here. Structurally, biofilms are macromolecular gels composed of high-molecular-weight polysaccharides, DNA, and proteins containing embedded cells (30). Macromolecular gels consisting of both proteins and carbohydrates have shown to have fairly large pore sizes that allow submicrometer particles to diffuse freely (41). Thus, micelle-encapsulated eugenol and carvacrol appear to be good vehicles to deliver hydrophobic antimicrobials through a potentially complex gel structure of expolymeric substances to the bacterial membrane of embedded cells. However, the biofilms grown on stainless steel coupons were treated by submersion in the antimicrobial-loaded micelle solution under static conditions. We suggest that additional forced convection, e.g., by shaking the solution containing the submerged chips during the antimicrobial exposure treatment, may lead to an improved contact of the loaded surfactant micelles with the biofilm, which could improve efficacies further. Potentially, the developed system could in the future be used in combination with other treatments to remediate biofilms from food and food contact surfaces if solely generally recognized as safe components are used in their manufacture.

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