Antibacterial and antibiofilm activities of thyme oil against foodborne multiple antibiotics-resistant *Enterococcus faecalis*

Fang Liu,[*,†] Panpan Jin,[*] Hansheng Gong,[‡] Zhilan Sun,[*] Lihui Du,[§,1] and Daoying Wang[*,1]

[*]*Institute of Agricultural Products Processing, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China;* [†]*School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, China;* [‡]*School of Food Engineering, Ludong University, Yantai 264025, China; and* [§]*College of Food Science and Engineering, Collaborative Innovation Center for Modern Grain Circulation and Safety, Key Laboratory of Grains and Oils Quality Control and Processing, Nanjing University of Finance and Economics, Nanjing 210023, China*

ABSTRACT The inhibitory and bactericidal activities of thyme oil against the foodborne multiple antibiotics-resistant *Enterococcus faecalis* biofilm were evaluated in this study. Gas chromatography-mass spectrometry revealed that more than 70% of the composition of thyme oil is thymol. Crystal violet staining assay showed that 128 and 256 μg/mL thyme oil significantly inhibited the biofilm formation of *E. faecalis*. The cell adherence of *E. faecalis*, as shown by its swimming and swarming motilities, was reduced by thyme oil. The exopolysaccharide (**EPS**) quantification assay showed that thyme oil inhibited the EPS synthesis in *E. faecalis* biofilms. The 3D-view observations through confocal laser scanning and scanning electron microscopy suggested that cell adherence and biofilm thickness were decreased in thyme oil–treated biofilms. Quantitative real-time analyses showed that the transcription of *ebp* and *epa* gene clusters, which were related to cell mobility and EPS production, was inhibited by thyme oil. Thus, thyme oil effectively inhibited the biofilm formation of *E. faecalis* by affecting cell adherence and EPS synthesis. Furthermore, 2,048 and 4,096 μg/mL thyme oil can effectively inactivate *E. faecalis* population in the mature *E. faecalis* biofilms by 5.75 and 7.20 log CFU/mL, respectively, after 30 min of treatment. Thus, thyme oil at different concentrations can be used as an effective antibiofilm or germicidal agent to control *E. faecalis* biofilms.

Key words: plant essential oil, biofilm, *Enterococcus faecalis*, exopolysaccharide, cell adherence

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INTRODUCTION

Enterococci are gram-positive bacteria which are natural components of the intestinal flora of humans and many animals (Foulquié Moreno et al., 2006; Rehaiem et al., 2016; Chajęcka-Wierzchowska et al., 2017). They can easily survive for long periods and can contaminate the animal carcass and processed products (Foulquié Moreno et al., 2006; Rehaiem et al., 2016; Chajęcka-Wierzchowska et al., 2017). Moreover, two *Enterococcus* species (*Enterococcus faecalis* and *Enterococcus faecium*) are important opportunistic pathogens and caused a wide variety of infections including endocarditis, urinary tract infections, prostatitis, intra-abdominal infection, cellulitis, and wound infection as well as concurrent bacteremia, which mainly occurred among hospitalized patients (Gao et al., 2018; Fiore et al., 2019). Enterococci have intrinsic resistance to antibiotics and can form biofilms on abiotic surfaces (Holmberg and Rasmussen, 2016; Qayyum et al., 2018). A biofilm is a community of matrix-enclosed microorganisms that adhere to a surface (Costerton et al., 1995). The bacterial cells in biofilms are protected by the extracellular matrix of proteins, polysaccharides, and nucleic acids. As a result, these bacterial cells are more resistant to antibiotics or antibacterial agents than planktonic cells (Whitehead and Verran, 2015; Liu et al., 2017). Enterococci biofilms that form in food-processing environments are difficult to eliminate, which make enterococci as one of the common opportunistic pathogens and spoilage bacteria in meat products (Giaouris et al., 2014; Pesavento et al., 2014; Rizzotti et al., 2016; Liu et al., 2020). Therefore, novel, safe, and

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[1]Corresponding authors: ddabc_2000@163.com (LD); daoyingwang@yahoo.com (DW)

effective antibiofilm agents that can inhibit the formation of enterococci biofilm during food processing and storage should be developed.

Plant essential oils are a class of natural antimicrobial compounds with good antimicrobial activities against bacteria, viruses, and fungi (Wattanasatcha et al., 2012; Marchese et al., 2016; Zhang et al., 2016; Cui et al., 2019). Secondary metabolites with a phenolic structure are the main antimicrobial compounds in these plant essential oils (Wattanasatcha et al., 2012; Marchese et al., 2016). Thymol (2-isopropyl-5-methylphenol) is a monoterpene phenol found in the essential oils extracted from *Thymus*, *Moschus*, *Dracocephalum*, and *Origanum* (Wattanasatcha et al., 2012; Marchese et al., 2016). Thyme oil possesses strong inhibitory activity against many foodborne pathogens or spoilage bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *E. faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio alginolyticus*, and *Salmonella typhimurium* (Wattanasatcha et al., 2012; Marchese et al., 2016; Miladi et al., 2016). The application of thymol will expand if it exhibits good antibiofilm activity against foodborne pathogens at concentrations lower than the minimum inhibitory concentration. In this work, the changes in the biofilm formation of *E. faecalis* in the presence of thyme oil were studied using the crystal violet biofilm and 3D morphological analyses. The possible molecular mechanism of its inhibitory action was further studied by analyzing the changes of cell motility, exopolysaccharide (**EPS**) production, and the expression levels of genes related to biofilm formation. Finally, the effects of thyme oil at concentrations higher than the minimal inhibitory concentration (**MIC**) to inactivate *E. faecalis* cells in mature biofilms were also evaluated.

MATERIAL AND METHODS

Bacterial Strain and Culture Condition

E. faecalis R612Z1 was isolated from the product of Chinese water-boiled salted duck (Liu et al., 2020) and naturally resistant to multiple antibiotics, including vancomycin, erythromycin, streptomycin sulfate, cephalexin, tetracycline, ampicillin, and kanamycin. This bacterium was stored in a refrigerator at −80°C and inoculated in a brain heart infusion (**BHI**) broth (Beijing Land Bridge Tech. Co., Beijing, China) at 37°C for approximately 6 h to obtain the logarithmic phase cells for subsequent studies.

Chemical Composition of Thyme Oil

Thyme oil was extracted from thyme by Hubei Xinrunde Chemical Industry Co., Ltd. (Wuhan, China). The composition analysis of thyme oil was determined using gas chromatography-mass spectrometry (**GC-MS**) (Thermo Scientific TSQ 8000 EVO). The sample was separated using the Thermo Scientific TraceGOLD TG-5MS GC (30 m × 0.25 mm × 0.25 μm). GC-MS was performed using an electron ionization system at 70 eV, and the injector and detector temperatures were maintained at 280°C. The temperature setting of the chromatographic column was 40°C for 1 min and 280°C for 2 min (heating rate = 6°C min^{-1}). The diluted samples (1.0 mL, 1/100 (v/v) in methanol) were injected manually in splitless mode. The individual components in thyme oil were identified on the basis of their retention times and through the comparison of mass spectra with entries in IST105 and Wiley7.0 libraries. Each component percentage was calculated on the basis of the GC peak area via the normalization method. For all experiments, thyme oil was dissolved in absolute ethanol to obtain a stock solution of 20,480 μg/mL and twice diluted with BHI broth or sterile distilled water to obtain a series of thyme oil dilutions.

Determination of MIC

Each well in the 96-well microliter plate was added with 125 μL 100-diluted logarithmic phase *E. faecalis* culture of approximately 7 log CFU/ml and added with thyme oil solution diluted with 125 μL of BHI broth, obtaining final concentrations of 16, 32, 64, 128, 256, 512, and 1,024 μg/mL. The bacteria were further cultured at 37°C, and cell growth was monitored at 600 nm by using an Infinite M200 Microplate Reader (Tecan, Mannedorf, Switzerland). The lowest concentration of thyme oil that inhibited the visible growth of *E. faecalis* was designated as the MIC value.

Determination of Antibiofilm Activity

The antibiofilm activity of thyme oil against *E. faecalis* was evaluated in accordance with previously described methods (Sandasi et al., 2010; Bazargani and Rohloff, 2016; Liu et al., 2019). The 100-diluted logarithmic phase *E. faecalis* cultures of approximately 7 log CFU/mL were cultured in BHI broth in a 96-well microplate for 72 h at 37°C and supplied with thyme oil at final subminimal concentrations of 0, 64, 128, and 256 μg/mL. The BHI broth with or without thyme oil was changed every 24 h. At predetermined sampling times (12, 24, 48, and 72 h), the biofilms in the plate were dried and stained with 0.1% crystal violet for 30 min. After removing the excess dyes, the biofilms were destained using 150 μL of 95% ethanol, and the $OD_{570\ nm}$ values were measured by using a multimode plate reader (Infinite M200 PRO).

Changes in Cell Motility

The changes in the cell motility of *E. faecalis* after adding thyme oil were analyzed in accordance with previously described procedures (Cong et al., 2011; Hidalgo et al., 2011). Two agars for swimming (10 g/L tryptone, 5 g/L NaCl, 2.5 g/L glucose, and 0.3% agar) and swarming (25 g/L LB, 0.5 g/L glucose, and 0.5% agar) analyses were used. The 3-μL 100-diluted logarithmic phase *E. faecalis* culture was spotted on the agar plate surfaces containing varying concentrations of thyme oil (0, 64,

Table 1. Primer sequences for real-time PCR.

Gene	Primers	Sequence (5′-3′)
Housekeeping	*gdhA*	Forward 5′-GGAATTGATGTGGCGTTAG-3′ Reverse 5′-GTGTTGCACATGGTAACG-3′
Endocarditis and biofilm-associated Ebp pilus subunits	*ebpA*	Forward: 5′-CTACGACACTCTTGCTGGAA-3′ Reverse: 5′-AATTTCGCTCCTTTAAGTGG-3′
	ebpB	Forward: 5′-AACAGAAATTCCTTTTACGG-3′ Reverse: 5′-ATAGCCAAATGACTACCAGC-3′
	ebpC	Forward: 5′-TTGAAGTGGTGACAGGTGGG-3′ Reverse: 5′-GCTGCTTTCGTTGTTTCATC-3′
Epa polysaccharide synthesis	*epaA*	Forward: 5′-TTGCATCACCGCTTGTTATC-3′ Reverse: 5′-TCGCCAACTAGACCGATTAG-3′
	epaB	Forward: 5′-CGGATACAGAAACAACGGAT-3′ Reverse: 5′-TAGAGAATCCGATAGCCTGC-3′
	epaE	Forward: 5′-CGAAGTCAAATTACGCAGTG-3′ Reverse: 5′-AGGATTCGTGTGTGCCTGTA-3′
	epaG	Forward: 5′-CCGATGGTGAAGAAGACAAT-3′ Reverse: 5′-TGATTGTTTCTGCCAAGCCT-3′
	epaH	Forward: 5′-TCCAAGAAATCCTGGACGAC-3′ Reverse: 5′-CAAATGCCATAAATTCGGCT-3′
	epaM	Forward: 5′-GAGATACGATGGACGTGACT-3′ Reverse: 5′-TGCTTTACCATTTCTAAGGG-3′
	epaQ	Forward: 5′-TGGTTTTGTCGGAGTAGCTG-3′ Reverse: 5′-CCACAAAAGGTTCCATACCG-3′
	epaR	Forward: 5′-AATTTAG`AACGATGTCGGCA-3′ Reverse: 5′-CCGACAATGGACATATCTCC-3′

128, and 256 μg/mL). The diameters of bacterial growth after incubation at 30°C for 8 or 20 h were measured for swimming or swarming motilities.

Changes in EPS

The contents of EPS in *E. faecalis* biofilms were detected in accordance with previous studies (Harimawan and Ting, 2016; Liu et al., 2020). The logarithmic phase *E. faecalis* cultures were 100-fold diluted in BHI broth with or without thyme oil (0, 64, 128, and 256 μg/mL). A 1-mL cell suspension was added into 24-well plates and incubated for 3 D at 37°C. The BHI broth with or without thyme oil was changed every 24 h. The content of each well was harvested after the addition of 1 mL of phosphate-buffered saline (**PBS**) after washing and drying of the biofilm samples. The contents of ten wells represented ten replicates for one treated concentration and were mixed and centrifuged for 30 min at 5,000 g and 4°C. The concentrated precipitates were resuspended in 10-mL aqueous solution (0.85% NaCl, 0.22% formaldehyde) for 30 min at 80°C, and the EPS dissolved in the formaldehyde solution was extracted by centrifugation for 30 min at 15,000 *g* and 4°C. The concentrations of extracted EPS solutions were quantified using the phenol-sulfuric acid (**PSA**) method according to the previous articles (Dubois et al., 1956; Jiang et al., 2013). Briefly, the 5% phenol solution was mixed with 98% concentrated sulfuric acid in volume ratio of 1:5. One milliliter of standard and EPS solutions was transferred into microcentrifuge tubes, and 5 mL of the prepared PSA reagent was added. The test tubes were placed in a water bath at 100°C for 15 min, and the absorbance were measured at 490 nm using a multimode plate reader (Infinite M200 PRO).

Confocal Laser Scanning Microscopy

The biofilms grown in the presence of thyme oil were observed through confocal laser scanning microscopy (**CLSM**) (Liu et al., 2017; 2018). The logarithmic phase *E. faecalis* cultures were 100-fold diluted in BHI broth with or without thyme oil (0, 64, 128, and 256 μg/mL) and cultivated in 8-well chamber slides (Nunc Lab-Tek; Fisher Scientific) at a volume of 400 μL/well at 37°C for 3 D. The BHI broth with or without thyme oil was changed every 24 h. The biofilms in the wells were washed with 0.01-M PBS and dyed using the LIVE/DEAD BacLight viability kit (Molecular Probes; Life Technologies, Eugene, OR) at indicated sampling times (12, 24, 48, and 72 h). The samples were visualized using the Leica Ultra View VOX CLSM (Leica Microsystems, Ltd., Wetzlar, Germany) and analyzed using the Volocity software (Improvision; PerkinElmer, Cambridgeshire, UK). The excitation and emission wave lengths of SYTO 9 and PI were 485 and 498 nm and 535 and 637 nm, respectively.

Scanning Electron Microscopy

The bacterial cell microstructures of *E. faecalis* biofilms were visualized through scanning electron microscopy (**SEM**). The biofilms were cultured similar to that for CLSM and fixed with 2.5% glutaraldehyde at 4°C for 30 min at the indicated sampling times (12, 24, 48, and 72 h). The slide was cut in different parts corresponding to each sample, and the fixed samples were dehydrated using a graded ethanol series of 25, 50, 70, 90, and 100% for 10 min. The final biofilm samples were examined through SEM (EVO-LS10; Zeiss,

Table 2. Chemical compositions of thyme oil used in this study.

Composition	Formula	Proportion (%)
Decane, 4-methyl-	$C_{11}H_{24}$	0.12%
Hexane, 3,3-dimethyl-	C_8H_{18}	0.20%
Decane, 2-methyl-	$C_{11}H_{24}$	0.17%
Thymol	$C_{10}H_{14}O$	70.76%
Decane, 3-ethyl-3-methyl-	$C_{13}H_{28}$	1.48%
Nonadecane, 2-methyl-	$C_{20}H_{42}$	0.82%
1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy) tetrasiloxane	$C_{12}H_{36}O_4Si_5$	22.42%
Heptasiloxane, hexadecamethyl-	$C_{16}H_{48}O6Si_7$	1.71%
1-Decanol, 2-hexyl-	$C_{16}H_{34}O$	1.06%
Total		98.74

Oberkochen, Germany) at an accelerating voltage of 20 kV and magnification of 5,000×.

Isolation of RNA and Quantitative Real-Time Reverse Transcription PCR

The changes in biofilm-related genes in transcriptional levels were analyzed using quantitative real-time reverse transcription PCR (**qRT-PCR**). The control and thyme oil–treated *E. faecalis* biofilms were cultured in 24-well plates at 37°C for 24 h. The total RNA from the bacterial cells in biofilms was isolated using the TIANamp RNAprep pure Cell/Bacteria kit (Tiangen, China). The isolated RNAs were reverse-transcribed into cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, China). The transcription levels of the selected biofilm-related genes in Ebp pili and Epa polysaccharide clusters were analyzed using the SYBR Premix Ex Taq II (Takara, China) in the Roche LightCycler 480 Real-Time PCR System (Indianapolis, IN). The primers of the selected genes are listed in Table 1. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Liu et al., 2020). All samples were analyzed in triplicate.

Bactericidal Activities of Thyme Oil Against Bacterial Cells in Mature E. faecalis Biofilms

The bactericidal effect of thyme oil at concentrations higher than MIC against the *E. faecalis* bacterial cells in mature biofilms was evaluated. The *E. faecalis* biofilms cultivated in 24-well plates for 72 h were used and treated with different concentrations of thyme oil (512, 1,024, 2,048, and 4,096 μg/mL) for 5, 15, 30, and 60 min (Liu et al., 2017). After treatment, sterile water (negative control) or thyme oil solution was removed from the wells, and the biofilms were washed using 0.01-M PBS. The bacterial cells in each well were suspended in 9 mL of 0.01-M PBS buffer and added with 9 mL of 0.01-M PBS. The diluted 100-μL cell suspension was cultured on BHI plates in duplicate and incubated at 37°C for bacterial enumeration.

Statistical Analysis

All experiments were performed in triplicate, and results were presented as mean ± standard deviation. A statistical analysis except for qRT-PCR data was carried out by ANOVA by using the SPSS software (version 19.0; IBM-SPSS Inc., Armonk, NY). $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Identification of the Active Components of Thyme Oil

The active compositions of the thyme oil used in this study were analyzed via GC-MS, and the result is

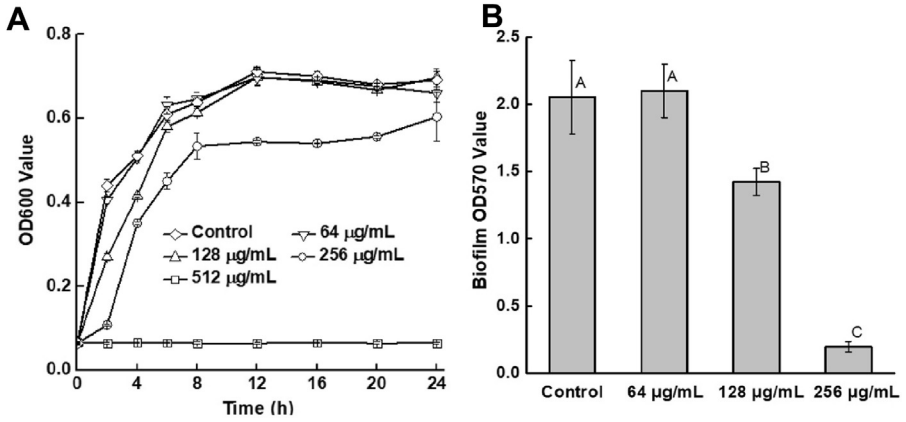

Figure 1. Effects of different concentrations of thyme oil on planktonic cell growth (A) and biofilm formation (B) of *Enterococcus faecalis* R612-Z1. The different capital letters in the figures indicate different treatments with significant differences ($P < 0.05$).

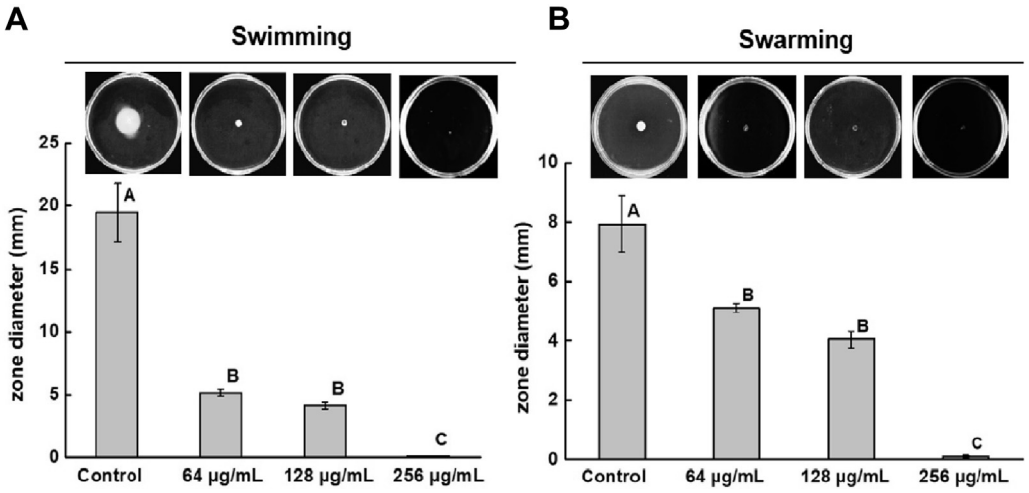

Figure 2. Effects of different concentrations of thyme oil on cell swimming (A) and swarming (B) motilities of *Enterococcus faecalis* R612-Z1. The different capital letters in the figures indicate the significant differences in different treatments ($P < 0.05$).

presented in Table 2. Nine components representing 98.74% of the total detected constituents were identified, which included thymol (70.76%), 1,1,1,3,5,5,7,7,7-Nonav methyl-3-(trimethylsiloxy) tetrasiloxane (22.42%), heptasiloxane, hexadecamethyl- (1.71%), decane, 3-ethyl-3-methyl- (1.48%), and 1-Decanol, 2-hexyl- (1.06%). The result suggested thymol was the predominant component of thyme oil used in this study.

Antibiofilm Activity of Thyme Oil Against E. faecalis

The results of *E. faecalis* R612-Z1 growth curves under different concentrations of thyme oil are shown in Figure 1A. The MIC value of thyme oil was 512 μg/mL, which was the concentration in which the bacterial cells did not grow. The antibiofilm activity of 64, 128,

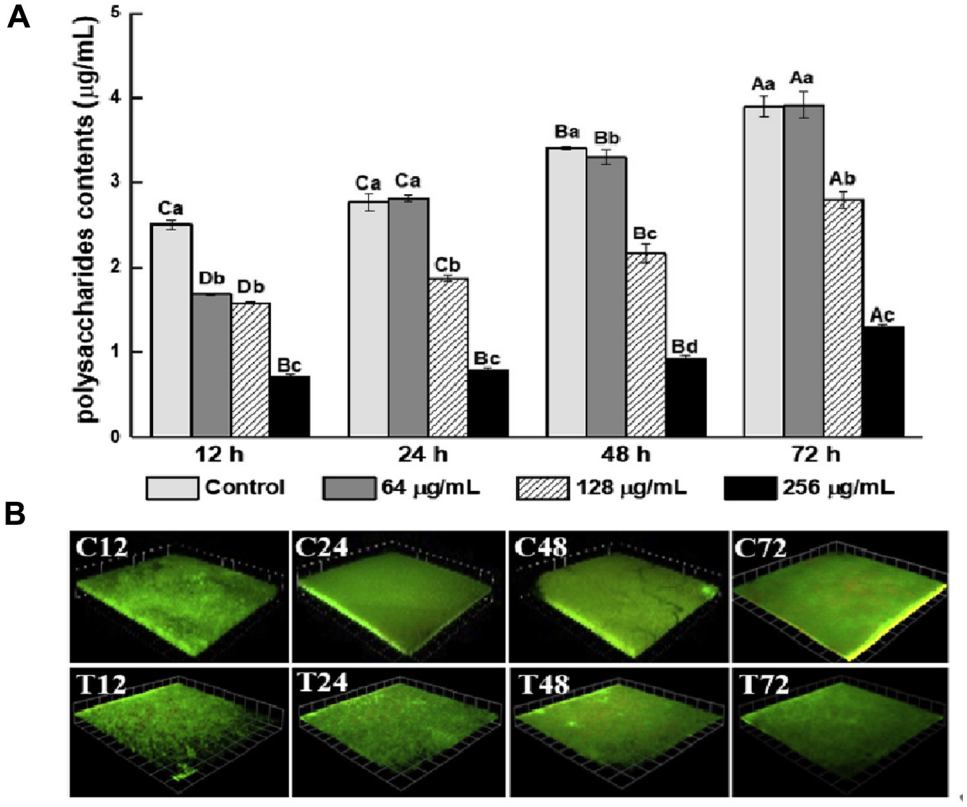

Figure 3. Change in the polysaccharide contents (A) and microstructure (B) of *Enterococcus faecalis* biofilms. The polysaccharide contents were detected by phenol-sulfuric acid method. The 3D images of confocal laser scanning microscopy corresponding to the microstructure and height of *E. faecalis* biofilms were analyzed using the *E. faecalis* R612-Z1 biofilms grown at 37°C for 12 (C12, T12), 24 (C24, T24), 48 (C48, T48), and 72 h (C72, T72). C, controls; T, 128 μg/mL thyme oil–treated groups. The different capital letters in the figures indicate that the different treatments had significant differences ($P < 0.05$). The different small letters in the figures indicate significant differences in the treatments ($P < 0.05$).

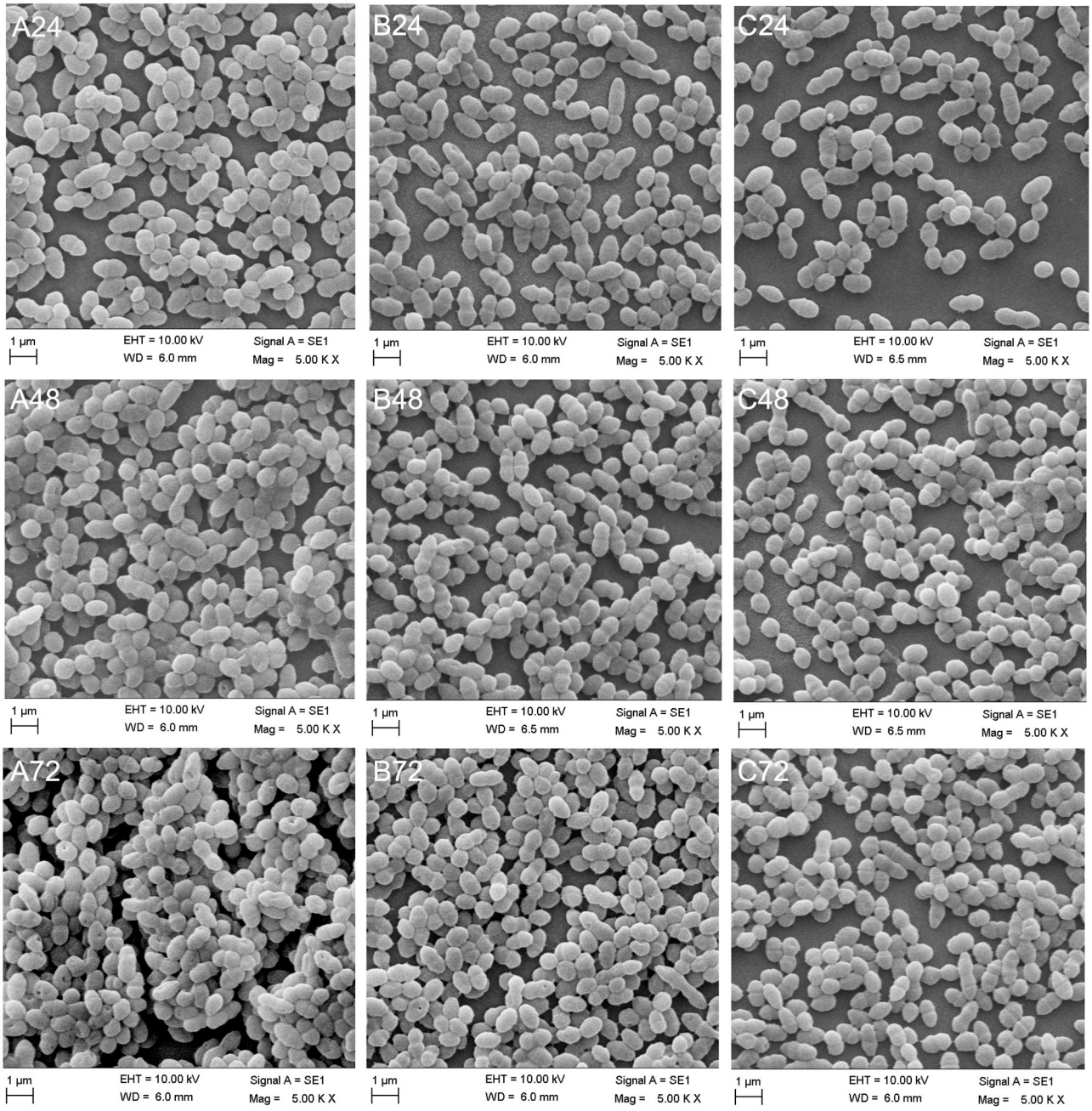

Figure 4. SEM images of *Enterococcus faecalis* R612-Z1 biofilms grown at 37°C for 24 (A24, B24, C24), 48 (A48, B48, C48), and 72 h (A72, B72, C72). A, control; B, 128 μg/mL thyme oil–treated group; C, 256 μg/mL thyme oil–treated group.

and 256 μg/mL thyme oil against *E. faecalis* R612-Z1 was detected through crystal-violet biofilm assays. As shown in Figure 1B, the biofilm formation of *E. faecalis* after 72 h of growth was decreased significantly by 128 and 256 μg/mL thyme oil ($P < 0.05$) (Figure 1B).

Foodborne pathogens that form biofilms in food processing environments are difficult to eliminate. Many studies have focused on the antibiofilm activities of plant oils against foodborne pathogens and spoilage bacteria. Kim et al. (2016) reported that bay, clove, pimento berry oils, and their major common constituent eugenol are effective in inhibiting the *E. coli* O157:H7 biofilm formation without affecting planktonic cell growth. Lee et al. (2014) reported that the extracts of *Ginkgo biloba* inhibit *E. coli* O157:H7 and *S. aureus* biofilm formation on the surfaces of polystyrene and glass. Szczepanski et al. (2014) studied the antibiofilm activities of thyme, oregano, and cinnamon essential oil at sublethal concentrations on the biofilm formation of *Sphingomonas*, *Acinetobacter*, and *Stenotrophomonas* and found that thyme oil is more effective in inhibiting biofilm development than the other two oils. Nithyanand et al. (2015) reported that *Pogostemon* oil can inhibit the growth of streptococcal biofilms. These antibiofilm plant oils are plant secondary metabolites that contain phenolics. The present study showed that thyme oil can also inhibit the biofilm formation of *E. faecalis* effectively.

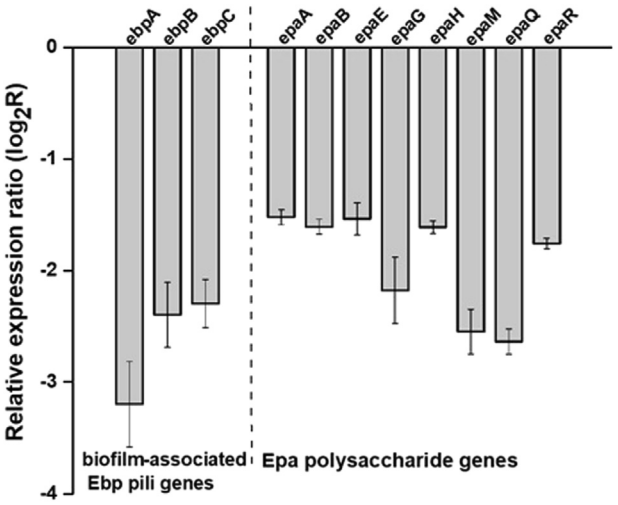

Figure 5. Changes in the transcription levels of genes (*ebpA*, *ebpB*, *ebpC*, *epaA*, *epaB*, and *epaE*) in the two clusters of *ebp* and *epa* in *Enterococcus faecalis* R612-Z1 biofilms grown at 37°C for 24 h after treatment with 128 μg/mL thyme oil.

Effect of Thyme Oil on Cell Motility of E. faecalis

Bacterial adhesion plays a main role in the initial stage of biofilm formation, and bacterial cell motility can affect this adhesion (Borges et al., 2012; Monte et al., 2014). Some nonflagellated cocci, including *S. aureus* and *E. faecalis*, are historically regarded as nonmotile bacteria. In the recent years, some articles have reported that *S. aureus*, a nonflagellated bacterium, can spread across agar surfaces and is actively motile under certain conditions (Borges et al., 2012; Pollitt et al., 2015). The biofilm-associated Ebp pili of *E. faecalis* play an important role in bacterial adherence to different surfaces (La Rosa et al., 2016; Afonina et al., 2018). Besides Ebp pili, type IV Pili are also important in cell motility, adherence, and biofilm formation of *E. faecalis* (Kohler et al., 2018; Schmitt et al., 2018). The effects of thyme oil on *E. faecalis* motility were analyzed, and the diameters of bacterial halos corresponding to swimming and swarming motilities of bacterial cells treated with 64, 128, and 256 μg/mL thyme oil significantly decreased compared with those of the control ($P < 0.05$) (Figure 2). Thus, the cell motility of *E. faecalis* can be inhibited by thyme oil. The cell motility of *E. faecalis* may be due to the effect of bacterial surface pili, and the decrease of cell motility means the decrease of bacterial adherence. So, the antibiofilm activity of thyme oil may be partly attributed to the inhibition of bacterial adherence. Bai et al. (2019) reported that the cell motility of *S. aureus* is also inhibited by shikimic acid.

Other plant oils inhibit biofilm formation by interfering with bacterial motility. Merghni et al. (2018) reported that the *Eucalyptus globulus* essential oil and its main component 1,8-cineole can inhibit the swarming behavior of methicillin-resistant *S. aureus* strains. Lee et al. (2018) studied the antibiofilm activity of two different plant extracts and found their different inhibition activities against cell motility. Harmaline can reduce swarming motility, whereas norharmane has a remarkable inhibitory effect on swimming motility. As cell motility plays an important role in quorum sensing-mediated biofilm formation, these plant-oriented antibiofilm agents may interfere with bacterial quorum sensing at different levels to inhibit the cell swimming or swarming ability of bacteria.

Inhibition of Biofilm EPS Production by Thyme Oil

Bacteria can continue to grow and secrete the extrapolymeric biofilm matrix, which includes EPS, proteins, fatty acids, and nucleic acids, when they adhere to the solid surface (Flemming and Wingender, 2010). The extrapolymeric matrix accounts for 80% of biofilms and forms the mature biofilm architecture (Jung et al., 2013; Roy et al., 2018). EPS is the major component of the extrapolymeric matrix (Flemming and Wingender, 2010). Thus, the inhibition or reduction of EPS production is important in controlling biofilm formation.

The EPS content in the control or thyme oil–treated *E. faecalis* biofilms was detected via a quantification assay. As shown in Figure 3A, the EPS contents in *E. faecalis* biofilm were significantly inhibited by addition of 128 and 256 μg/mL thyme oil ($P < 0.05$) and can be potentially inhibited by addition of 64 μg/mL thyme oil. Thus, treatment with 128 and 256 μg/mL thyme oil can inhibit EPS production of *E. faecalis* cells in biofilms.

The 3D architecture changes corresponding to the contents of extrapolymeric matrix of *E. faecalis* biofilms in the presence of thyme oils were further visualized through CLSM. As shown in Figure 3B, the bacterial cells in control *E. faecalis* biofilms were closely adherent and formed well-organized structures at 12, 24, 48, and

Table 3. Bacterial counts in the biofilms of *E. faecalis* R612-Z1 grown on 24-well flat-bottom polystyrene plates at 37°C for 3 D after treated with thyme oils at different concentrations for 5, 15, 30, and 60 min.

	The bacterial counts in biofilms (log CFU/mL)				
Treatment time	Control	512 μg/mL	1,024 μg/mL	2,048 μg/mL	4,096 μg/mL
5 min	$9.39 \pm 0.19^{A,a}$	9.29 ± 0.15	$9.55 \pm 0.20^{A,a}$	$9.32 \pm 0.03^{A,a}$	$9.29 \pm 0.07^{A,a}$
15 min	$9.28 \pm 0.28^{A,a}$	$9.06 \pm 0.17^{A,a}$	$9.42 \pm 0.15^{A,a}$	$5.91 \pm 0.82^{B,b}$	$5.38 \pm 0.21^{B,b}$
30 min	$9.33 \pm 0.16^{A,a}$	$9.12 \pm 0.24^{A,a}$	$7.78 \pm 0.59^{B,b}$	$3.57 \pm 0.38^{C,c}$	$2.09 \pm 0.13^{C,d}$
60 min	$9.15 \pm 0.09^{A,a}$	$9.25 \pm 0.11^{A,a}$	$7.54 \pm 0.35^{B,b}$	$3.63 \pm 0.21^{C,c}$	$2.54 \pm 0.15^{C,d}$

The different capital letters in the figures indicate that the different treatments had significant differences ($P < 0.05$). The different small letters in the figures indicate significant differences in the treatments ($P < 0.05$).

72 h of incubation (Figures 3B–C12, C24, C48, and C72), whereas those treated with 128 μg/mL thyme oil had porous structures with uniform holes and decreased thickness (Figures 3B–T12, T24, T48, and T72). These biofilm images showed that the extrapolymeric matrix production of bacterial cells in biofilms was inhibited in the presence of thyme oil. This finding was in agreement with the results obtained via the EPS quantification assay. Chen et al. (2018) reported that a small molecule ST056083 can inhibit the secretion of EPS and biofilm formation of *E. faecalis*. Liu et al. (2019) reported that anionic chitosan can also inhibit EPS synthesis during the formation of *P. aeruginosa* biofilms.

Scanning Electron Microscopy

The aggregation states of bacterial cells in the biofilms at different cultivation stages during 24, 48, and 72 h of culture were observed through SEM. In Figure 4, the bacterial cells in the control biofilms aggregated densely and thickly, which were typical characteristics of biofilm formation. The dense aggregates of cells were reduced upon treatment with 128 and 256 μg/mL thyme oil. Thus, thyme oil can reduce bacterial cell-cell adhesion by inhibiting EPS production. This antibiofilm effect of thyme oil is in accordance with many other antibiofilm agents. Bai et al. (2019) reported that shikimic acid can make bacterial cells in *S. aureus* biofilms loose and discrete, suggesting the role of shikimic acid in preventing the attachment of bacterial cells.

Transcriptional Changes of Biofilm-Related Genes Induced by Thyme Oil

Bacterial pili and polysaccharide synthesis are important in different biofilm formation stages, including aggregation, adherence, and maturation (Montealegre et al., 2015; Afonina et al., 2018). The endocarditis and biofilm-associated pili encoded by *ebp* operon play an important role in the aggregation and adherence of *E. faecalis* (Nallapareddy et al., 2006; Sillanpää et al., 2013). The polysaccharide biosynthesis of *E. faecalis* depends on the *epa* operon (Rigottier-Gois et al., 2015; Dale et al., 2017). Thus, the differential expressions of *ebp* and *epa* operons were studied via qRT-PCR to investigate the genetic bases of *E. faecalis* R612-Z1 biofilm inhibition by thyme oils. RNAs were extracted from the bacterial cells in control and 128 μg/mL thyme oil–treated biofilms grown for 24 h. As shown in Figure 5, the expressions of the Ebp pili (*ebpABC*) and the Epa polysaccharide (*epaABEGHMQR*) genes were markedly inhibited 2- to 9-fold in the presence of 128 μg/mL thyme oil. Thus, thyme oil can inhibit biofilm formation by affecting the transcription of pili and polysaccharide genes.

Inactivation of Bacterial Cells in Mature Biofilms

The effectiveness of thyme oil at concentrations higher than MIC to inactivate *E. faecalis* cells in mature biofilms was further evaluated. The mature biofilms were cultivated on 24-well plates at 37°C for 3 D. The bacterial cells in the biofilms were treated with 512, 1,024, 2,048, and 4,096 μg/mL thyme oil for 5, 15, 30, and 60 min. Results are shown in Table 3. The initial *E. faecalis* counts in biofilms were approximately 9.3 log CFU/mL. The bacterial counts in the biofilms did not have significant differences when treated with selected thyme oil solutions for 5 min ($P > 0.05$). When the treatment time of 512 and 1,024 μg/mL thyme oil was prolonged to 15 min, the bacterial counts did not show any significant difference. However, the bacterial count was significantly decreased after treatment with 2,048 and 4,096 μg/mL thyme oil compared with that of the control ($P < 0.05$). The effectiveness of treatments by using 1,024, 2,048, and 4,096 μg/mL thyme oil was significantly increased when the treatment time was prolonged from 15 min to 30 min ($P < 0.05$) but did not increase with prolonged treatment time from 30 min to 60 min.

Based on the results, the bacterial cells in the biofilms were more difficult to inactivate than the planktonic bacterial cells. The difficulty of inactivating bacterial cells in biofilms may be due to the protection provided by the extrapolymeric matrix (Liu et al., 2017), reflecting the importance of increasing the germicidal efficacy of sanitizers to inhibit the secretion of this matrix. Foodborne pathogens are difficult to eliminate once they form biofilms on the surface of food equipment. Thus, effective methods must be designed to inhibit biofilm formation.

CONCLUSIONS

In this study, different concentrations of thyme oils were found to have effective antibiofilm or germicidal effects against *E. faecalis* R612-Z1 biofilms. Biofilm formation was significantly reduced after treatment with subinhibitory concentrations (128 and 256 μg/mL) of thyme oil. The antibiofilm effect of thyme oil was associated with cell motility reduction and EPS production. Thyme oil at concentrations of 2,048 and 4,096 μg/mL effectively inactivated the bacterial cells in 3-day-old mature biofilm of *E. faecalis* grown on 24-well plates after treatment for 30 min. This study revealed that thyme oil can be used as an effective green antibacterial agent in food processing.

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REFERENCES

Afonina, I., X. N. Lim, R. Tan, and K. A. Kline. 2018. Planktonic interference and biofilm alliance between aggregation substance and endocarditis- and biofilm-associated pili in *Enterococcus faecalis*. J. Bacteriol. 200 e00361-18.

Bai, J., K. Zhong, Y. Wu, G. Elena, and H. Gao. 2019. Antibiofilm activity of shikimic acid against *Staphylococcus aureus*. Food Control 95:327–333.

Bazargani, M. M., and J. Rohloff. 2016. Antibiofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms. Food Control 61:156–164.

Borges, A., M. J. Saavedra, and M. Simões. 2012. The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. Biofouling 8:755–767.

Chajęcka-Wierzchowska, W., A. Zadernowska, and Ł. Łaniewska-Trokenheim. 2017. Virulence factors of *Enterococcus* spp. presented in food. Lwt-food Sci. Technol. 75:670–676.

Chen, L., X. Li, X. Zhou, J. Zeng, Z. Ren, L. Lei, D. Kang, K. Zhang, J. Zou, and Y. Li. 2018. Inhibition of *Enterococcus faecalis* growth and biofilm formation by molecule targeting cyclic di-AMP synthetase activity. J. Endodont. 44:1381–1388.

Cong, Y. G., J. Wang, Z. J. Chen, K. Xiong, Q. W. Xu, and F. Q. Hu. 2011. Characterization of swarming motility in *Citrobacter freundii*. FEMS Microbiol. Lett. 317:160–171.

Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott. 1995. Microbial biofilms. Annu. Rev. Microbiol. 49:711–745.

Cui, H., C. Zhang, C. Li, and L. Lin. 2019. Antibacterial mechanism of oregano essential oil. Ind. Crop Prod. 139:111498.

Dale, J. L., J. L. Nilson, A. M. T. Barnes, and G. M. Dunny. 2017. Restructuring of *Enterococcus faecalis* biofilm architecture in response to antibiotic-induced stress. NPJ Biofilms Microbiomes 3:15.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350–356.

Fiore, E., D. V. Tyne, and M. S. Gilmore. 2019. Pathogenicity of enterococci. Microbiol. Spectr. 7 https://doi.org/10.1128/microbiolspec.GPP3-0053-2018.

Flemming, H. C., and J. Wingender. 2010. The biofilm matrix. Nat. Rev. Microbiol. 8:623–633.

Foulquié Moreno, M. R., P. Sarantinopoulos, E. Tsakalidou, and L. De Vuyst. 2006. The role and application of enterococci in food and health. Int. J. Food Microbiol. 106:1–24.

Gao, W., B. P. Howden, and T. P. Stinear. 2018. Evolution of virulence in *Enterococcus faecium*, a hospital-adapted opportunistic pathogen. Curr. Opin. Microbiol. 41:76–82.

Giaouris, E., E. Heir, M. Hébraud, N. Chorianopoulos, S. Langsrud, T. Møretrø, O. Habimana, M. Desvaux, S. Renier G, and J. Nychas. 2014. Attachment and biofilm formation by foodborne bacteria in meat processing environments: causes, implications, role of bacterial interactions and control by alternative novel methods. Meat Sci. 97:298–309.

Harimawan, A., and Y. P. Ting. 2016. Investigation of extracellular polymeric substances (EPS) properties of *P. aeruginosa*, and *B. subtilis*, and their role in bacterial adhesion. Colloids Surf. B Biointerfaces 146:459–467.

Hidalgo, G., M. Chan, and N. Tufenkji. 2011. Inhibition of *Escherichia coli* CFT073 *fliC* expression and motility by cranberry materials. Appl. Environ. Microbiol. 77:6852–6857.

Holmberg, A., and M. Rasmussen. 2016. Mature biofilms of *Enterococcus faecalis* and *Enterococcus faecium* are highly resistant to antibiotics. Diagn. Microbiol. Infect. Dis. 84:19–21.

Jiang, Q., and Y. Xie. 2013. Improvement of phenol sulfuric acid method for the determination of polysaccharides (in Chinese). Jiangsu Agricultural Sci 41:316–318.

Jung, J. H., N. Y. Choi, and S. Y. Lee. 2013. Biofilm formation and exopolysaccharide (EPS) production by *Cronobacter sakazakii* depending on environmental conditions. Food Microbiol. 34:70–80.

Kim, Y. G., J. H. Lee, G. Gwon, S. I. Kim, J. G. Park, and J. Lee. 2016. Essential oils and eugenols inhibit biofilm formation and the virulence of *Escherichia coli* O157:H7. Sci. Rep. 6:36377.

Kohler, V., W. Keller, and E. Grohmann. 2018. *Enterococcus* adhesin PrgB facilitates type IV secretion by condensation of extracellular DNA. Mol. Microbiol. 109:263–267.

La Rosa, S. L., M. C. Montealegre, K. V. Singh, and B. E. Murray. 2016. *Enterococcus faecalis* Ebp pili are important for cell-cell aggregation and intraspecies gene transfer. Microbiology 162:798–802.

Lee, J. H., Y. G. Kim, S. Y. Ryu, M. H. Cho, and J. Lee. 2014. Ginkgolic acids and Ginkgo biloba extract inhibit *Escherichia coli* O157:H7 and *Staphylococcus aureus* biofilm formation. Int. J. Food Microbiol. 174:47–55.

Lee, J. H., Y. G. Kim, S. H. Shim, and J. Lee. 2017. Antibiofilm activities of norharmane and its derivatives against *Escherichia coli* O157:H7 and other bacteria. Phytomedicine 3:254–261.

Liu, F., L. Du, T. Zhao, P. Zhao, and M. P. Doyle. 2017. Effects of phenyllactic acid as sanitizing agent for inactivation of *Listeria monocytogenes* biofilms. Food Control 78:72–78.

Liu, Y., Y. Jiang, J. Zhu, J. Huang, and H. Zhang. 2019. Inhibition of bacterial adhesion and biofilm formation of sulfonated chitosan against *Pseudomonas aeruginosa*. Carbohydr. Polym. 206:412–419.

Liu, F., Z. Sun, F. Wang, Y. Liu, Y. Zhu, L. Du, D. Wang, and W. Xu. 2020. Inhibition of biofilm formation and exopolysaccharide synthesis of Enterococcus faecalis by phenyllactic acid. Food Microbiol 86:103344.

Liu, F., F. Wang, L. Du, T. Zhao, P. M. Doyle, D. Wang, X. Zhang, Z. Sun, and W. Xu. 2018. Antibacterial and antibiofilm activity of phenyllactic acid against *Enterobacter cloacae*. Food Control 84:442–448.

Marchese, A., I. E. Orhan, M. Daglia, R. Barbieri, A. Di Lorenzo, S. F. Nabavi, O. Gortzi, M. Izadi, and S. M. Nabavi. 2016. Antibacterial and antifungal activities of thymol: a brief review of the literature. Food Chem. 210:402–414.

Merghni, A., E. Noumi, O. Hadded, N. Dridi, H. Panwar, O. Ceylan, M. Mastouri, and M. Snoussi. 2018. Assessment of the antibiofilm and antiquorum sensing activities of Eucalyptus globulus essential oil and its main component 1,8-cineole against methicillin-resistant *Staphylococcus aureus* strains. Microb. Pathog. 118:74–80.

Miladi, H., T. Zmantar, Y. Chaabouni, K. Fedhila, A. Bakhrouf, K. Mahdouani, and K. Chaieb. 2016. Antibacterial and efflux pump inhibitors of thymol and carvacrol against food-borne pathogens. Microb. Pathog. 99:95–100.

Monte, J., A. C. Abreu, A. Borges, L. C. Simões, and M. Simões. 2014. Antimicrobial activity of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. Pathogens 3:473–498.

Montealegre, M. C., S. L. La Rosa, J. H. Roh, B. R. Harvey, and B. E. Murray. 2015. The *Enterococcus faecalis* EbpA pilus protein: attenuation of expression, biofilm formation, and adherence to fibrinogen start with the rare initiation codon ATT. mBio 6 e00467-15.

Nallapareddy, S. R., K. V. Singh, J. Sillanpää, D. A. Garsin, M. Höök, S. L. Erlandsen, and B. E. Murray. 2006. Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. J. Clin. Invest. 116:2799–2807.

Nithyanand, P., R. M. B. Shafreen, S. Muthamil, R. Murugan, and S. K. Pandian. 2015. Essential oils from commercial and wild Patchouli modulate group A Streptococcal biofilms. Ind. Crop Prod. 69:180–186.

Pesavento, G., C. Calonico, B. Ducci, A. Magnanini, and A. Lo Nostro. 2014. Prevalence and antibiotic resistance of Enterococcus spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. Food Microbiol. 41:1–7.

Pollitt, E. J., S. A. Crusz, and S. P. Diggle. 2015. *Staphylococcus aureus* forms spreading dendrites that have characteristics of active motility. Sci. Rep. 5:17698.

Qayyum, S., D. Sharma, D. Bisht, and A. U. Khan. 2019. Identification of factors involved in *Enterococcus faecalis* biofilm under quercetin stress. Microb. Pathog. 126:205–211.

Rehaiem, A., I. Fhoula, A. F. Slim, I. B. B. Boubaker, A. B. Chihia, and H. Ouzaria. 2016. Prevalence, acquired antibiotic resistance and bacteriocin production of *Enterococcus* spp. isolated from tunisian fermented food products. Food Control. 63:259–266.

Rigottier-Gois, L., C. Madec, A. Navickas, R. C. Matos, E. Akary-Lepage, M. Y. Mistou, and P. Serror. 2015. The surface rhamnopolysaccharide *epa* of *Enterococcus faecalis* is a key determinant of intestinal colonization. J. Infect. Dis. 211:62–71.

Rizzotti, L., F. Rossi, and S. Torriani. 2016. Biocide and antibiotic resistance of *Enterococcus faecalis* and *Enterococcus faecium* isolated from the swine meat chain. Food Microbiol. 60:160–164.

Roy, R., M. Tiwari, G. Donelli, and V. Tiwari. 2018. Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action. Virulence 9:522–554.

Sandasi, M., C. M. Leonard, and A. M. Viljoen. 2010. The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. Lett. Appl. Microbiol. 50:30–35.

Schmitt, A., K. Jiang, M. I. Camacho, V. R. Jonna, A. Hofer, F. Westerlund, P. J. Christie, and R. P. A. Berntsson. 2018. PrgB promotes aggregation, biofilm formation, and conjugation through DNA binding and compaction. Mol. Microbiol. 109:291–305.

Sillanpää, J., C. Chang, K. V. Singh, M. C. Montealegre, S. R. Nallapareddy, B. R. Harvey, H. Ton-That, and B. E. Murray. 2013. Contribution of individual Ebp Pilus subunits of *Enterococcus faecalis* OG1RF to pilus biogenesis, biofilm formation and urinary tract infection. PLoS One. 8:e68813.

Szczepanski, S., and A. Lipski. 2014. Essential oils show specific inhibiting effects on bacterial biofilm formation. Food Control. 36:224–229.

Wattanasatcha, A., S. Rengpipat, and S. Wanichwecharungruang. 2012. Thymol nanospheres as an effective anti-bacterial agent. Int. J. Pharm. 434:360–365.

Whitehead, K. A., and J. Verran. 2015. Formation, architecture and functionality of microbial biofilms in the food industry. Curr. Opin. Food Sci. 2:84–91.

Zhang, Y., X. Liu, Y. Wang, P. Jiang, and S. Y. Quek. 2016. Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. Food Control 59:282–289.