A natural biopreservative: Antibacterial action and mechanisms of Chinese *Litsea mollis* Hemsl. extract against *Escherichia coli* DH5α and *Salmonella* spp.

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ABSTRACT

Chemical preservatives have potential safety hazards, which may pose threats to human health. Safer biopreservatives are therefore urgently required. This study investigated the bacteriostatic activity and mechanism of *Litsea mollis* Hemsl. essential oil against *Escherichia coli* DH5α and *Salmonella* spp. Antibacterial activity of *Litsea mollis* Hemsl. essential oil 9 (LMEO9) against *E. coli* DH5α was observed (zone of inhibition was 5.0 ± 0.2 mm; minimum inhibitory concentration was 0.05%). Increases in electrolyte, nucleic acid, and alkaline phosphatase leakage in LMEO9-treated bacteria suggested that the cell envelope had been damaged. Scanning and transmission electron microscopy also demonstrated morphological alterations and content leakage during LMEO9 treatment. According to the kill-time analysis and propidium iodide uptake assay, LMEO9 led to cell death. These results demonstrated that LMEO9, which could affect bacterial cell envelope structural integrity, is a low-cost biopreservative that could be useful for the dairy industry and in fresh storage.

Key words: antibacterial activity, *Litsea mollis* Hemsl. essential oil, mechanism of action, chemical composition

INTRODUCTION

Due to the existence of pathogenic microorganisms, food spoilage and food poisoning have led to huge economic losses and continued threats to human health (Yuan and Yuk, 2018). In the United States, 18 out-

breaks originating from organic foods occurred from 1992 to 2014 (56% of these outbreaks occurred from 2010 to 2014), accounting for 779 cases of illness, 258 hospital stays, and 3 deaths (Harvey et al., 2016). According to World Health Organization statistics (WHO, 2015), non-typhoidal *Salmonella* and diarrheal *Escherichia coli* are the major causes of foodborne disease. Several synthetic antimicrobial compounds, including potassium sorbate and nitrate, have been shown to prevent food pathogen growth (Zhang et al., 2017a). These additives increase the shelf life of various products and decrease foodborne disease outbreaks. However, due to cumulative toxicity and increasing antibiotic resistance to conventional antiseptics, the use of natural antibacterial agents has gained intense research interest (Bajpai et al., 2013). Plant-derived essential oils (EO) have been shown to possess antioxidant (Bonilla et al., 2018), antimycotic (Dudoiu et al., 2017), and antibacterial (Diao et al., 2013) activities. Because the majority of EO are certified as generally recognized as safe (GRAS) substances by the US Food and Drug Administration (FDA; Kamatou and Viljoen, 2010), they represent attractive prospects for safer food preservation applications.

Litsea mollis Hemsl. belongs to the Lauraceae family and is primarily distributed in southwestern China. *Litsea mollis* Hemsl. essential oils (LMEO) from flowers and leaves are significant sources of citral, an ingredient of food spice (Wu et al., 2008). The global supply of *Litsea* oils is sufficient, and, compared with nisin, LMEO has a better cost benefit (Saikia et al., 2013). Although the chemical composition and antimicrobial activity of Vietnamese LMEO have been elucidated (Son et al., 2014; Nguyen et al., 2018), little information as to the effects of Chinese LMEO on *Escherichia coli* and *Salmonella* is known. Thus, we analyzed the chemical composition of Chinese LMEO, its bacteriostatic effects, and its mechanisms of action. The addition of LMEO can extend the shelf lives of dairy products such as pasteurized milk.

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MATERIALS AND METHODS

Microbial Culture and Chemicals

Nine strains of bacteria were used: 5 gram-negative strains (*Salmonella* spp., *Pseudomonas aeruginosa* ZFM1, *Escherichia coli* O104, *E. coli* O157, and *E. coli* DH5 α), and 4 gram-positive strains (*Enterococcus faecalis* BM13, *Listeria monocytogenes* J4045, *L. monocytogenes* Clip11262, and *Staphylococcus aureus* ZFM1). All bacterial strains were provided by the Key Laboratory for Food Microbial Technology of Zhejiang Province (Hangzhou, China). Strains were stored in Microbank vials (Pro-Lab Diagnostics Inc., Richmond Hill, ON, Canada) at -80°C and cultured at 37°C in Luria-Bertani (LB) medium or brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK). All chemicals were of analytical grade.

Essential Oil Extraction

Three dried fruits of *Litsea mollis* Hemsl. from Wanzhou city, Chongqing Province, China, were ground using a micro-plant grinder, and the powder was hydro-distilled for 5 h using a Clevenger-type apparatus (Transparent Acrylic Glove Box, Plexiglass Lab Equipment, Jiangsu, China). Following extraction, LMEO was dried over anhydrous sodium sulfate, sealed, and preserved in the dark at 4°C before analysis. The EO were termed LMEO4, LMEO9, and LMEO28, respectively, according to the geographical area of origin of each sample.

Analysis Using GC-MS

Following dissolution in ethanol (5 μL) and dehydration with anhydrous sodium sulfate, LMEO constituents were determined using TRACE GC Ultra gas chromatography (Thermo Fisher Scientific, Waltham, MA) in a column (60 m $\times$ 0.25 mm; 0.25- μm film thickness) and mass selective detector (Polaris Q-Ion Trap MS). The mass selective detector was set to electron impact ionization (230°C) mode, using a mass scan range of 50 to 500 m/z at 70 eV. The carrier gas was helium (99.999%), at 1.5 mL/min flow rate and a 1:10 split ratio. Injector and mass spectrometry transfer line temperatures were 220°C and 250°C , respectively. The initial temperature of 50°C was increased to 170°C at 3°C per minute, topping out at 250°C for 3 min. The EO composition was identified through a comparison of the GC retention index with standard specimens, statistics in the published literature, and the National Institute of Standards and Technology 08 Mass Spectral Library and Mass Spectral Search Program. The

peak area was used to quantify the relative percentages of the separated compounds.

Antibacterial Assays

Determination of Bacterial Inhibition Zones.

A slightly modified disk diffusion method served to quantify the diameter of inhibition zone (DIZ), as previously described (Diao et al., 2014a). Briefly, cultures were grown at 37°C for 24 h in LB liquid medium, after which 200- μL inoculums (approximately 1×10^6 cfu/mL) and 10 mL of semi-solid medium were uniformly spread onto solid LB medium. Oxford cups were placed onto plates, and pure LMEO (50 μL) was added. Plates were left at 37°C for 24 h, and DIZ values (mm) were measured with a ruler. Control treatment used was broth LB.

Determination of MIC and Minimum Bactericidal Concentrations.

Minimum inhibitory concentrations and minimum bactericidal concentrations (MBC) were assessed using the microdilution method with some modifications (Wang et al., 2017). Briefly, LMEO was thoroughly dissolved in absolute ethanol to obtain serial dilutions in concentrations ranging from 1 to 100%. Bacterial suspensions were added to obtain a final EO concentration of 0.01 to 1%. Control tests were performed using 0.99% ethanol. The MIC represented the lowest concentration of EO at which no bacterial growth was visible after 24 h of cultivation at 37°C . Suspensions that exhibited no visible growth were spread onto LB medium plates and subcultured at 37°C for 24 h. The MBC represented the lowest concentration of EO at which no surviving colonies were observed.

Kill-Time Analysis

The LMEO9 possessed the broadest bacteriostatic spectrum and highest antibacterial activity. *Escherichia coli* DH5 α and *Salmonella* spp. were selected as the model microorganisms for subsequent experiments because of higher sensitivity to LMEO9. Cells in the exponential growth phase were exposed to MIC and MBC of LMEO9 and cultivated for 24 h at 37°C with uniform shaking. Bacterial viability was quantified every 4 h through absorbance measurements at 600 nm, performed on a microplate reader (Spectra Max 190, Molecular Devices LLC, San Jose, CA; Zengin and Baysal, 2014).

Alkaline Phosphatase Leakage Assays

Cell wall integrity was assessed through alkaline phosphatase (AKP) leakage assays (Song et al., 2018).

For AKP assessment, bacteria were cultured overnight, and the inoculum was centrifuged at $6,000 \times g$ (4°C) for 8 min. Bacterial pellets were rinsed with PBS (pH 7.2), resuspended through vortexing, and washed 3 more times. Bacterial solutions were mixed with different concentrations (MIC and MBC) of LME09, and extracellular AKP levels were measured using a commercial AKP kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. The control was 0.99% ethanol.

Membrane Damage Assessment

Relative Electrical Conductivity Assays. Membrane permeability was assessed through relative electrical conductivity (**REC**) using an electrical conductivity (**EC**) meter (Mettler-Toledo International Inc., Zurich, Switzerland; Diao et al., 2014b). For these assays, bacteria were cultured overnight and pelleted by centrifugation at $6,000 \times g$ (4°C) for 8 min. The cell suspension was flushed with 5% glucose until it reached the same EC as the glucose solution. Isotonic bacteria were then treated with different concentrations of LME09 (control, MIC, and MBC), and the EC of each mix was identified as L_1 . Bacteria were then cultured at 37°C for 12 h, with EC measured every 2 h, marked as L_2 . The EC of bacteria in the same glucose solution treated in boiling water for 5 min was designated L_0 . The REC was quantified as follows:

$$\text{REC (\%)} = 100 \times (L_2 - L_1) \div L_0.$$

Nucleic Acid Leakage Assays. The extracellular concentration of nucleic acids indicates cell membrane integrity (Mutlu-Ingok and Karbancioglu-Guler, 2017). For nucleic acid assays, bacteria were pelleted by centrifuging at $6,000 \times g$ for 8 min (4°C) and resuspended in PBS. These experimental steps were repeated in triplicate. Harvested suspensions (500 μL) were treated with various concentrations of LME09 (control, MIC, and MBC) at 37°C , and cultures were collected at 4-h intervals. Optical density at 260 nm of supernatant was measured on a microplate reader (Spectra Max 190, Molecular Devices LLC).

Propidium Iodide Staining Assays

Propidium iodide (**PI**) uptake is a standard cell death marker due to its ability to stain DNA by penetrating the cell membranes of dead or dying cells (Madhuri et al., 2009). Cell survival was assessed using a PI reagent kit (BBI Life Sciences Co., Ltd., Shanghai, China). Bacteria cultures that had been grown overnight were

pelleted by centrifugation at $6,000 \times g$ for 8 min, rinsed in PBS twice, and finally resuspended in assay buffer. We added PI staining solution and LME09 to bacterial suspensions, followed by incubation at 37°C for 5 to 30 min. We assessed PI staining via fluorescence microscopy (Oplenic Optronics Co., Jiangsu, China).

Microscopic Observations

Scanning Electron Microscopy. Bacterial morphology was observed using scanning electron microscopy following LME09 treatment. For scanning electron microscopy assessment, overnight cultures (5 mL) were collected and treated with LME09 (control, MIC, MBC). Suspensions were centrifuged at $4,000 \times g$ for 10 min and fixed with 2.5% (vol/vol) glutaraldehyde solution overnight at 4°C . Cells were pelleted, rinsed in PBS for 15 min 3 times, and treated with 1% osmic acid for 1.5 h. Washed cells were dehydrated in ethanol (30 to 100%) and dried in liquid carbon dioxide. Following cathodic spraying with platinum, morphological observations were assessed by scanning electron microscopy (Hitachi TM1000, Hitachi Ltd., Tokyo, Japan).

Transmission Electron Microscopy. Treatments were performed as for scanning electron microscopy, and samples were dehydrated in 30, 50, 80, 90, and 95% ethanol for 15 min, followed by exposure to 100% ethanol and 100% acetone, each for 20 min. Following hydration, an embedding reagent was added to the specimen for 12 h at 70°C . Stained cells were imaged using a transmission electron microscope (Hitachi H-7650, Hitachi Ltd.).

Statistical Analysis

All experiments were performed in triplicate. Data shown are mean $\pm$ standard deviation (SD). Significance between groups was evaluated using one-way ANOVA and Duncan's test. The cutoff for statistical significance set at $P < 0.05$.

RESULTS AND DISCUSSION

Chemical Composition of LME0

The composition of LME0 was identified by GC-MS (Supplemental Table S1: <https://doi.org/10.3168/jds.2019-16292>). The main components of LME04 were neral (46.66%), followed by geranial (36.52%). Likewise, LME09 and LME028 were mainly composed of neral (36.64 and 42.44%, respectively) and geranial (37.20 and 48.95%, respectively), similar to LME0 derived from Taiwan (Ho et al., 2010) and southern regions of

Table 1. Bacterial growth inhibition zones¹ (mm) of *Litsea mollis* Hemsl. essential oils (LMEO) from 3 regions of China

Bacterial strain	LMEO4	LMEO9	LMEO28
Gram negative			
<i>Salmonella</i> spp.	4.1 ± 0.9 ^a	4.2 ± 0.2 ^a	3.6 ± 0.8 ^a
<i>Escherichia coli</i> O157	4.0 ± 0.6 ^b	2.6 ± 0.5 ^b	4.6 ± 0.7 ^b
<i>E. coli</i> DH5α	3.0 ± 0.6 ^c	5.0 ± 0.2 ^c	3.0 ± 0.6 ^c
<i>E. coli</i> O104	0	1.4 ± 0.3 ^d	1.2 ± 0.2 ^d
<i>Pseudomonas aeruginosa</i> ZFM1	0	0	0
Gram positive			
<i>Enterococcus faecalis</i> BM13	0	0	0
<i>Listeria monocytogenes</i> J4045	0	0	0
<i>L. monocytogenes</i> Clip 11262	0	0	0
<i>Staphylococcus aureus</i> ZFM1	0	0	0

^{a-d}Different lowercase superscripts in the same columns represent statistically significant differences between means ($P < 0.05$).

¹Diameters of inhibition zones were determined by modified disk diffusion method. Values indicate the average ± SD of experiments performed in triplicate. Diameter of inhibition zone values have been calculated minus the diameter of Oxford cup (8 mm).

China (Wang and Liu, 2010). The antibacterial activity of the compounds was ranked as follows: phenol > aldehyde > ketone > alcohol > ether > hydrocarbon (Nguyen et al., 2018). Aldehyde was the dominant component of LMEO, which presumably contributes to its noticeable antibacterial abilities as a food preservative (Li et al., 2013; Sun et al., 2015). A different inhibitory activity against *E. coli* was observed between LMEO (MIC 0.01% to 0.5%) and Indian Lauraceae EO (MIC approximately 0.003 to 0.02%), which is mainly composed of sabinene, and Vietnamese LMEO (MIC 0.003 to 0.006%, 1,8-cineole or linalool type) was more potent against *Salmonella* Typhimurium compared with its Chinese counterpart (MIC 0.5%). This was due to less noticeable bacteriostatic effect of citral than that of sabinene, 1,8-cineole, and linalool (Saikia et al., 2013; Nguyen et al., 2018).

Determination of Antibacterial Efficacy

The DIZ values of LMEO were determined using the disk diffusion method. The highest bacteriostatic effect

was observed in LMEO9 (Table 1), with the highest levels of antimicrobial activity against *E. coli* DH5α (5.0 mm) and *Salmonella* spp. (4.2 mm). Against *E. coli* O157 and *E. coli* O104, LMEO9 displayed moderate activity, with DIZ values of 2.6 and 1.4 mm, respectively. Against *E. faecalis* BM13, *L. monocytogenes* J4045, *L. monocytogenes* Clip 11262, *Staph. aureus* ZFM1, and *P. aeruginosa* ZFM1, LMEO9 displayed no inhibitory activity. Thus, LMEO9 primarily inhibits gram-negative bacteria.

Measurements of MIC and MBC are displayed in Table 2. The MIC of *Salmonella* and *E. coli* O157 were 0.5 and 0.1%, whereas the MBC was 1% for both bacterial strains. Against *E. coli* DH5α, LMEO9 displayed potent antimicrobial activity, with an MIC value of 0.05% and an MBC of 0.1%. The efficacy of *Litsea* EO against *E. coli* O104 was negligible.

Antibacterial screening results show the inhibition of primarily gram-negative bacteria by LMEO9. *Pseudomonas aeruginosa* showed no bacteriostatic activity, consistent with previous studies that found *Pseudomonas* to be the least sensitive to EO (Burt, 2004). The

Table 2. Activity of *Litsea mollis* Hemsl. essential oils (LMEO) from 3 regions of China against gram-negative bacteria; results determined by modified microdilution method

Bacterial strain	LMEO4		LMEO9		LMEO28	
	MIC ¹ (%)	MBC ² (%)	MIC (%)	MBC (%)	MIC (%)	MBC (%)
<i>Salmonella</i> spp.	0.5	1	0.5	1	0.5	1
<i>Escherichia coli</i> O157	0.01	0.05	0.1	1	0.05	0.1
<i>E. coli</i> DH5α	0.05	0.1	0.05	0.1	0.5	1
<i>E. coli</i> O104	>1	>1	>1	>1	>1	>1

¹MIC (% vol/vol) = minimum inhibitory concentration.

²MBC (% vol/vol) = minimum bactericidal concentration.

Figure 1. Effects of *Litsea mollis* Hemsl. essential oil (LMEO9) on viability of *Escherichia coli* DH5α (A) and *Salmonella* spp. (B). Cell viability was assessed by absorbance measurements of optical density at 600 nm (OD₆₀₀), recorded on a molecular microplate reader (Spectra Max 190, Molecular Devices LLC, San Jose, CA). Control group (squares) represents the bacteria exposed to 0.99% ethanol. Minimum inhibitory concentration (MIC, circles) and minimum bactericidal concentration (MBC, triangles) represent bacterial levels following treatments of LMEO9 at corresponding concentrations. Data are expressed as mean $\pm$ SD, analyzed using one-way ANOVA and Duncan's test; $P < 0.05$ was the cutoff for statistical significance.

bacteriostatic activity of LMEO may have been due to cell wall disruption (Nazzaro et al., 2013). Many studies currently suggest that, compared with gram-positive bacteria, gram-negative bacteria may be more resistant to EO because the high levels of cell wall LPS in gram-negative bacteria could inhibit influx of hydrophobic compounds (Calo et al., 2015). However, studies suggest that some EO are more active against gram-negative bacteria (Devi et al., 2010; Meng et al., 2016; Zhang et al., 2017a). These differences may be attributable to the varying constituents of EO and to higher levels of intracellular ATP leaking into the incubation solution following inhibitor exposure (Cui et al., 2015). Additionally, LMEO exhibits different bacteriostatic activity against *E. coli* O104, *E. coli* O157, and *E. coli* DH5α. The monoterpenes contained in EO exert antimicrobial effects that are dependent on membrane lipid composition and the net surface charge derived from LPS for gram-negative bacteria (Saikia et al., 2013). The diversity of the O-antigen in LPS allows it to act across *E. coli* strains, which may partly explain its varying potency (DebRoy et al., 2011; Shashkov et al., 2015). Furthermore, the composition values of neral and geranial are similar between EO (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-16292>). In spite of previous conclusions that oxygenated monoterpenes account for the majority of the antibacterial activity of *Litsea* (Saikia et al., 2013), our results suggest that other small fractions of components also affect EO activity. Kalemba and Kunicka (2003) concluded that ratios of constituents, rather than their quantities, account for the efficacy of EO.

Kill-Time Curves

According to the susceptibility of the pathogens, *E. coli* DH5α and *Salmonella* served as model microbes for

Figure 2. Effect of *Litsea mollis* Hemsl. essential oil (LMEO9) on damage to cell wall of *Escherichia coli* DH5α and *Salmonella* spp. Extracellular alkaline phosphatase (AKP) activity was determined using a commercial AKP kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Control group (unshaded bars) represents the bacteria exposed to 0.99% ethanol. Minimum inhibitory concentration (MIC, diagonal stripes) and minimum bactericidal concentration (MBC, cross-hatching) represent the strains following treatments of LMEO9 at corresponding concentrations. Data are expressed as mean $\pm$ SD, analyzed using one-way ANOVA and Duncan's test; $P < 0.05$ was the cutoff for statistical significance.

Figure 3. Effect of *Litsea mollis* Hemsl. essential oil (LMEO9) on cell membrane permeability of *Escherichia coli* DH5α (A) and *Salmonella* spp. (B). The relative electrical conductivity (REC) was measured using a Mettler Toledo (Columbus, OH) conductivity meter. Control group (squares) represents the bacteria with 0.99% ethanol treatment. Minimum inhibitory concentration (MIC, circles) and minimum bactericidal concentration (MBC, triangles) represent the strains following treatments of LMEO9 at corresponding concentrations. Data are expressed as mean $\pm$ SD, analyzed using one-way ANOVA and Duncan's test; $P < 0.05$ was the cutoff for statistical significance.

further exploration into the mode of action of LMEO9. Compared with controls, the number of viable *E. coli* DH5α exposed to the MIC of LMEO9 declined slightly during the first 4 h of treatment and then remained constant (Figure 1A). Unlike the variation trend of optical density at 600 nm at MIC, in treatments at MBC, a decrease of 59.5% from 1.501 to 0.608 over the first 16 h of treatment occurred, followed by a more gradual decrease over time. The loss of viability of *Salmonella* (Figure 1B) was not statistically significantly different from that of *E. coli*.

These data strongly suggest that LMEO9 is a natural preservative with bactericidal effects that are dependent on the time of exposure and concentration. Similar to these findings, *Litsea mollis* leaf EO from Vietnam have been shown to inhibit bacterial growth (Nguyen et al., 2018).

Cell Wall Damage Evaluation

Alkaline phosphatase cannot be detected in bacterial culture medium unless the cell wall has been destroyed

Figure 4. Effect of *Litsea mollis* Hemsl. essential oil (LMEO9) on cell membrane integrity of *Escherichia coli* DH5α (A) and *Salmonella* (B). The concentration of extracellular nucleic acid was determined by absorbance measurements at 260 nm (OD₂₆₀), recorded on a molecular microplate reader (Spectra Max 190, Molecular Devices LLC, San Jose, CA). Control group (squares) represents the bacteria exposed to 0.99% ethanol. Minimum inhibitory concentration (MIC, circles) and minimum bactericidal concentration (MBC, triangles) represent the strains following treatments of LMEO9 at corresponding concentrations. Data were expressed as mean $\pm$ SD, analyzed using one-way ANOVA and Duncan's test; $P < 0.05$ was the cutoff for statistical significance.

(Xu et al., 2016). The activity of AKP significantly increased (approximately 7-fold) when bacteria were treated with LMEO9 at MIC (Figure 2). In addition, the LMEO9 at MBC produced a larger increase in AKP compared with MIC, with the largest effects observed in *Salmonella* (0.1439 to 3.5709 U/mL). The increased AKP activity confirmed that LMEO9 damaged the bacterial cell wall. Therefore, LMEO9 exerts its bactericidal effect through a loss of cell wall integrity, allowing microbes and hydrolytic enzymes to enter cells, inducing cell death (Wang et al., 2017).

Cell Membrane Damage Evaluation

Cell Membrane Permeability Analysis. Bacterial membrane permeability was assessed using electrolyte leakage assays (Figure 3). Although control cells displayed a small increase in REC due to normal cell death, the REC of experimental groups displayed sig-

nificantly elevated values within the first 2 h, which then stabilized (22.6 and 18.1% for *E. coli* DH5 α and *Salmonella*, respectively) by 8 h. We found that K⁺, Na⁺, and Ca²⁺ had leaked from cells, indicating membrane permeability due to LMEO9. Ion leakage disrupts the electrochemical gradients required for ion channel activity and their associated metabolic functions, bringing about cell death (Patra et al., 2015). *Alpinia gunianensis*-leaf EO has similarly been shown to induce abnormal release of small molecules from bacterial cells (Deng et al., 2016).

Cell Membrane Integrity Analysis. Cytoplasmic membrane integrity was further assessed through nucleic acid leakage assays. The absorbance values measured at 260 nm rapidly increased over the first 4 h of LMEO9 treatment and reached sustained levels at 20 to 24 h (Figure 4). The time required to achieve an equilibrium of nucleic acids was longer than that of other small molecules, which reveals the ability of LMEO to de-

Figure 5. Cell viability of *Escherichia coli* DH5 α under microscopic observation (400 $\times$ magnification). Fluorescence was determined using a propidium iodide (PI) reagent kit (BBI Life Sciences Co. Ltd., Shanghai, China); fluorescence indicates bacterial death. Optical image of (A) untreated *E. coli* DH5 α ; and fluorescent images of (B) untreated *E. coli* DH5 α , (C) *E. coli* DH5 α treated with *Litsea mollis* Hemsl. essential oil (LMEO9) at MIC; and (D) *E. coli* DH5 α treated with LMEO9 at minimum bactericidal concentration (MBC).

stroy cytomembrane continually. (Figure 3). Additionally, LME09 generated more obvious alterations to the optical density at 260 nm at MBC compared with that caused by LME0 at MIC. These data could be due to cell membrane rupture and nucleic acid release into the medium in the presence of LME09. Loss of intracellular nucleic acids leads to decreased bacterial DNA replication, transcription, and translation, ultimately leading to cell death (Zhang et al., 2017a).

Fluorescence Microscopy Viability Evaluation

Propidium iodide is a fluorescent dye that can access the interior of dead cells and stain DNA (Zhang et al., 2017c). An increase in PI fluorescence intensity,

indicating the presence of dead bacteria, was observed in the presence of increasing concentrations of LME09 (Figures 5C, D). Optical (Figure 5A) and fluorescent microscope (Figure 5B) images revealed that untreated *E. coli* DH5 α appeared healthy in the culture medium. Thus, LME09 treatment induced bacterial cell death, most notably at higher concentrations, supporting the data from kill-time curves. Similar bactericidal effects were observed for curcumin I against *E. coli* (Tyagi et al., 2015).

Morphological Transformation

Images from scanning electron microscopy confirmed extensive morphological alterations to *E. coli* DH5 α

Figure 6. Morphological alterations in cell surface of *Escherichia coli* DH5 α and *Salmonella*. Observation was performed using scanning electron microscopy (35,000 $\times$ magnification). (A) Untreated *E. coli* DH5 α ; (B) *E. coli* DH5 α treated with *Litsea mollis* Hemsl. essential oil (LME09) at minimum bactericidal concentration (MBC); (C) untreated *Salmonella*; (D) *Salmonella* treated with LME09 at MBC.

and *Salmonella*. Untreated cells (Figure 6A and C) were regular, rod shaped, and appeared intact, whereas LMEO9 at MBC (Figure 6B and D) led to pore formation, cell lysis, and release of intracellular materials. Following LMEO9 treatment, extensive structural changes, including surface collapse and heterogeneous cytosol, were observed, as illustrated in Figure 7B and D. Transmission electron microscopy micrographs of the control group showed a dense cytoplasm and characteristic cell morphology (Figure 7A and C). Thus, microscopic evaluations revealed that LMEO9 exerted bactericidal activity by influencing cell envelope integrity, consistent with our cell wall and membrane damage data. Various plant extracts display a similar ability to induce physical changes in bacterial cell walls and membranes (Shan et al., 2007; Tyagi et al., 2015; Yuan et al., 2015). However, LMEO9 produced more notable morphological alterations in *E. coli* than those

observed for black pepper EO under identical treatment conditions (Zhang et al., 2017b).

CONCLUSIONS

We demonstrated that LMEO displays strong bacteriostatic effects against *E. coli* DH5 α and *Salmonella* spp. The antimicrobial activity of LMEO was elicited through physical and morphological damage of the bacteria cell envelope. We observed AKP, electrolyte, and nucleic acid release into the extracellular medium following LMEO9 treatment. These alterations led to loss of cell viability, supported by results from kill-time curves and fluorescent images. Analysis via GC-MS showed the main components of LMEO4, LMEO9, and LMEO28 to be citral (geranial and neral). The antibacterial activity of LMEO at lower level of neral is better than those containing more neral. To apply Chi-

Figure 7. Intracellular alterations to *Escherichia coli* DH5 α and *Salmonella*. Observation was performed using transmission electron microscopy (TEM; Hitachi, Tokyo, Japan). (A) Untreated *E. coli* DH5 α ; (B) *E. coli* DH5 α treated with *Litsea mollis* Hemsl. essential oil (LMEO9) at minimum bactericidal concentration (MBC); (C) untreated *Salmonella*; (D) *Salmonella* treated with LMEO9 at MBC.

nese LMEO to the biopreservative field, investigation of its synergism with other antimicrobial compounds, interaction with food compositions, and toxicological evaluations are now required.

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