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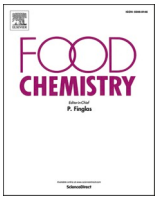

Emulsions containing composite (clove, oregano, and cinnamon) essential oils: Phase inversion preparation, physicochemical properties and antibacterial mechanism

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ABSTRACT

Natural essential oils (EOs), especially those combining different individual EOs (also termed composite EOs) with enhanced performance, are becoming healthy, market-sought food preservatives/additives. This study aims to provide insights into the challenge regarding EOs processing due to their low solubility and the elusive mechanism under the enhanced bio-reactivity of composite EOs. A unique oil/water interacting network was created by phase-inversion processing, which enhances EO solubilization and emulsification to form composite EO formulations (EOFs) containing ordinary cinnamon, oregano and clove EOs. These EOFs mainly contained cinnamaldehyde, carvacrol and eugenol and exhibited excellent post-storage stability. The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging ability of EOFs (at 15.880 μL/mL) was > 88%, and the Ferric reducing antioxidant power (FRAP) was 1.8 mM $FeSO_4{\cdot}7H_2O$. The minimum inhibitory concentration (MIC) of EOFs against *E. coli* and *S. aureus* was ~7.940 μL/mL. The EOFs could cause quick deterioration of bacterial structures, demonstrating high efficacy in bacteria-killing and anti-biofilm formation.

1. Introduction

Pathogenic bacteria refer to microorganisms that cause human or animal diseases, posing a major challenge to human health and societal safety. Pathogens can contaminate food directly or indirectly during processing and transportation. With food or drinking water as the medium, food poisoning and infectious diseases prevail worldwide. According to a 2022 WHO report, around one in ten people get sick from eating contaminated foods (WHO, 2022), with *Vibrio cholera* and *Escherichia coli* as the most common pathogens, which cause 420,000 annual deaths (children under five accounting for one-third) (Lan et al., 2022).

Food preservatives have been widely used for centuries, with many benefits or capacities to prevent food deterioration and poisoning, extend shelf life and reduce food costs. Recent years have seen a huge increase in using low-cost synthetic or artificial additives (antioxidants or antimicrobials) for food preservation. Although many of them are approved for moderate consumption by the authorities, their safety and potential harm are still controversial (Brewer, Sprouls, & Russon, 1994; Cohen Mitchell, 1992). For example, additives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are indicated as carcinogens, while some are linked to food allergies and inflammation (Bauer et al., 2005; Kelley & Gleason, 2016). Not surprisingly, nature-derived alternatives are sought by consumers and food companies, particularly with increasing living standards and health awareness. Consequently, there is a need and foreseeable market size for safe, high-performance and cost-effective natural additives (Mesias, Martin, & Hernandez, 2021).

Essential oils (EOs) are a group of oily substances containing volatile odorant compounds extracted from plant organs. Many EOs are innate antibacterial and antifungal agents and have antioxidant properties, with the main chemical components of aldehydes, phenols, alcohols, acids, ketones, terpenes and aromatic compounds (Li et al., 2022). Cinnamaldehyde in cinnamon oil, carvacrol in oregano oil, and eugenol

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in clove oil are often identified as the main components of EOs with antibacterial activity (Dogruyol, Mol, & Cosansu, 2020; Liu et al., 2021; Yoo, Baek, Heo, Yong, & Jo, 2021). However, EO products, especially those containing a single EO, normally fail to output broad-spectrum antibacterial activity, limiting further applications (Mangalagiri, Panditi, & Jeevigunta, 2021). From this point of view, developing advanced candidates containing multiple EOs is promising, as confirmed by recent studies. For example, it has been reported that using various EOs can synergistically enhance the antibacterial effects (Chraibi, Fadil, Farah, Lebrazi, & Fikri-Benbrahim, 2021). Purkait, Bhattacharya, Bag, and Chattopadhyay (2020) evaluated the efficacy of three commonly used EOs. Results showed that the cinnamon/clove oil combination had a synergistic effect on some bacteria and fungi. Dehghani, Hosseini, Golmakani, Majdinasab, and Esteghlal (2018) also found a synergistic effect between clove and Shirazi thyme EO emulsions, which could improve the quality of fillets. Similarly, some reported that the curcumin-clove oil emulsion has synergistic antibacterial effects, which effectively extend the shelf-life of fresh meat by 3 days (Zhao et al., 2022). Although the synergistic antibacterial mechanism of EOs is complex and rarely reported, some studies have attributed it to the presence of flavonoid compounds (Álvarez-Martínez, Barrajón-Catalán, Herranz-López, & Micol, 2021). Therefore, further exploring and thus enhancing the synergistic antibacterial effect of cinnamon, oregano, and clove EOs is of great importance.

However, EOs are volatile and hydrophobic, inherently limiting their processing and applications in aqueous solutions, particularly for composite EOs with varying properties and functions (Burt, 2004). An ideal solution is to use a solvent emulsifier to mix different EOs. Ferraz et al. (2021) mixed cinnamon and paprika oleoresins with varying proportions of whey protein isolate, gum Arabic or maltodextrin to obtain a more efficient and stable emulsion. Agrimonti, White, Tonetti, and Marmiroli (2019) released the active constituents in EOs in a more controlled fashion, which applied cellulosic pads, amended with emulsions containing oregano, thyme and cinnamon EOs to beef. Besides, EOs have the characteristics of solid volatility and poor durability, limiting their actual applications. The film-forming technique has been proven effective for EO processing and perseverance, thus enhacing their bactericidal effects. Chen et al. (2021) showed that edible chitosan film containing 0.15% oregano or 0.60% cinnamon EOs extended the shelf-life of roast duck for 7 days. In addition, cinnamon EO Pickering emulsion based on zein-pectin was found to have good storage stability and better antibacterial properties (Jiang, Wang, Li, Li, & Huang, 2020).

Herein, this study aims to investigate the preparation and bactericidal applications of emulsions containing combined ordinary (clove, oregano and cinnamon) EOs, obtain stable EO emulsions with improved performance on bacteria-killing and anti-biofilm formation and reveal the bactericidal mechanisms. EO emulsions with enhanced water solubility and stability was first prepared by the phase inversion of the blend of three ordinal EOs, i.e., clove, oregano and cinnamon oils. This strategy is used to prepare emulsion systems to disperse in liquid products and increase their antibacterial activity, thereby reducing their level of use to attenuate unpleasant sensory properties. Next, two groups of optimum EO formulations (EOFs) were obtained to evaluate their bacteria-killing and anti-biofilm formation activity against *E. coli* and *S. aureus*. Finally, the physicochemical properties, storage stability and antibacterial mechanism of EOFs were systematically characterized and analyzed. We expect this study to provide useful guidelines for developing next-generation, natural food preservatives.

2. Materials and methods

2.1. Materials

Cinnamon (*Cinnamomum zeylanicum L.*), oregano (*Origanum vulgare L.*), and clove (*Eugenia caryophyllata*) oils were purchased from the DoTERRA brand (Pleasant Grove, Utah, USA). Tween-80 was purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). LB broth, Mueller-Hinton agar (MHA), MacConkey (MAC) agar, Baird Parker (BP) agar, thiosulfate citrate bile salts sucrose (TCBS) agar and iron agar (IA) were purchased from Haibo Biotechnology Co., Ltd. (Qingdao, Shangdong, China). Other reagents used in the study were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All chemicals used were of analytical grade.

2.2. Bacterial strains and growth conditions

The bacterial strains used in this study were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 26112, *Vibrio parahaemolyticus* ATCC 17802 and *Bacillus subtilis* CMCC 63501 obtained from the Zhoushan Institute for Food and Drug Control. *Shewanella putrefaciens* BNCC 337021 was obtained from the Key Laboratory of Health Risk Factors for Seafood of Zhejiang Province. The strains were maintained as frozen stocks at -80 °C in protective beads before being plated onto the corresponding selective media: MAC agar, BP agar, TCBS agar, and IA, and incubated overnight at 37 °C to obtain single colonies before storage at 4 °C.

After 18 ± 2 h incubation, the cultures were diluted in LB or LB + 3 wt% NaCl (for *V. parahaemolyticus*) to an optical density of 0.1 at 600 nm, using a spectrophotometer (U-2800 spectrophotometer, Hitachi, Japan) to obtain the initial number of the bacterium at approximately 8-log CFU/mL. Upon use, a fresh bacterial suspension was diluted to 7-log CFU/mL.

2.3. Formulation of essential oils emulsion

2.3.1. Agar disc diffusion method of essential oils

The essential oils emulsion was prepared by the phase inversion method according to Cui, Wang, Wang, Li, and Zhang (2018) with some modifications. Briefly, cinnamon, oregano, or clove oil was combined with a solvent (containing 5% Tween-80, 10% ethanol, and water) at 7.5%, 5%, 2.5%, 1.25%, and 0%. Subsequently, the mixture was mixed by a vortex mixer for 30 s, rested for 10 s, and then mixed for 30 s to obtain the final EOs emulsion. The antibacterial potency of the EOs (cinnamon, oregano, and clove oils) was measured using the agar disc diffusion method, referring to a previous study (Hu et al., 2021). Each sterile petri plate (90 mm) was prepared with 20 mL MHA (3.5% NaCl to support the growth of *V. parahaemolyticus*). After solidifying, 100 μL of bacterial suspensions (7-log CFU/mL) were inoculated into the petri dishes containing MHA. The EO solutions (25 μL) were added to the medium quantitatively in five different holes. Afterward, the bacterial strains were incubated at 37 °C for 24 h. The diameter of the inhibition zone (DIZ) was obtained by measuring the range of the sterile growth zone in agar caused by essential oil diffusion. The minimum essential oil concentration that completely inhibits the growth of bacteria is the minimum inhibitory concentration (MIC). The experiment was performed in triplicates at each concentration.

2.3.2. Preparation of essential oils emulsion

The orthogonal method was adopted to obtain the best EOs formula, with the EO type as the factor and the EO concentration as the level. According to the MIC of each EO mentioned above (preliminary experiment, data not shown), three different concentrations were taken as the levels around the MIC (the same EO had different MIC for different bacteria), and a three-factor three-level experiment was set up (shown in Table S1, Supporting Information). Nine EO formulations were obtained by the orthogonal method. The EOs of the nine formulations were used to conduct bacteriostatic experiments on five experimental strains to get the best essential oil formulations (EOFs).

2.4. Minimum inhibitory concentration of EOF

The bacterial strains in this study represented Gram-negative (*E. coli*)

and Gram-positive bacteria (*S. aureus*), respectively, facilitating comparisons with other studies. The MIC of EOFs on bacteria was determined according to the standard broth microdilution method with some modifications (Lee, Kim, Beuchat, Kim, & Ryu, 2020). The EOFs were prepared to obtain final concentrations of 63.52 μL/mL, 31.76 μL/mL, 15.88 μL/mL, 7.94 μL/mL, 3.75 μL/mL, 1.985 μL/mL, 0.993 μL/mL, and 0.496 μL/mL in LB. The negative control (the solvent-only group) consisted of a bacterial suspension in LB and diluted solvent (containing 5% Tween-80, 10% ethanol, and water) without the EOs emulsion. Simultaneously, the bacterial suspension in LB was used as the blank control group. The concentration of EO with no visible bacteria on the microtiter plate after culturing at 37 °C for 24 h was considered the MIC.

2.5. Characterization of the EOF

2.5.1. Headspace gas chromatography-mass spectrometry (GC–MS) analysis

The GC–MS analysis was performed, referring to the method by Fancello et al. (2020), with some modifications. The EOF was analyzed using an Agilent 7890B gas chromatography system equipped with an Agilent 7697A autosampler. An Agilent 7000C triple quadrupole mass spectrometer system in electron ionization mode (EI) was used to identify the components of EOF. The headspace equilibration temperature was 50 °C, with a 20 min equilibration time, then 1 mL of the headspace gas was injected and analyzed by GC–MS. The chromatographic separation was performed on a DB-5MS capillary column (60 m × 0.25 μm × 0.25 mm) (Agilent, Agilent Technologies, USA). The temperature was kept at 40 °C for 1 min, and then Helium was used as the carrier gas at a flow rate of 1 mL/min. Finally, EOF chemical components were identified by comparing the mass spectra (matching with commercial and built-in libraries). The relative peak areas of specific compounds to the whole were used to reflect the relative contents of the volatile substances in the EOFs.

2.5.2. EOF physicochemical properties measurement

EOFs were prepared and stored in the dark at 25 °C. After 0 and 7 d of storage, the emulsion properties of EOFs, including droplet size, polydispersity index (PDI) and zeta potential, were determined according to Yu et al. (2017) using a nanoscale potentiometer (ZS90; Zetasizer Nano, UK). EOFs diluted 300 times with distilled water were used for the particle size analysis. The diluted emulsions were poured into a 1 cm polystyrene cell cuvette, which was then placed in the particle size analyzer. The measurement temperature was 25 °C, with an equilibration time of 2 min and a scattering angle of 90°. Each sample was measured three times in parallel with the averaged value used for the report.

10 mL of the sample was added to a clean beaker for pH assessments at 25 °C ± 1 °C using a calibrated pH meter (PHS-3C, INESA Scientific Instrument Co., Ltd., China). The color values of EOFs were evaluated with a Precise Colorimeter (CS-210, CHN Spec Technology Co., Ltd., China). The color was expressed in L* (lightness), a* (redness), and b* (yellowness). The samples were poured into clean 35 mm polystyrene plastic dishes and allowed to equilibrate. The measurements were made at different locations on each sample and averaged. All measurements were repeated at least three times.

2.6. Antioxidant activity evaluation

2.6.1. DPPH radical scavenging ability

Free radical scavenging activity of the EOs emulsion was measured with a DPPH Free Radical Scavenging Capacity Assay Kit (JianCheng Bioengineering Institute, Nanjing, China) according to the kit protocol and the experimental method of Zhang, Chen, Zhang, and Kang (2023). After the sample was mixed with the working solution, the mixture was incubated in the dark at 25 °C for 30 min and then centrifuged at 4000 rpm for 5 min. The Trolox solution was used as a standard. The free radical scavenging ability was determined at 517 nm by a UV–Vis spectrophotometer (U2800, Hitachi, Japan).

2.6.2. Ferric reducing antioxidant power (FRAP)

Under acidic conditions, antioxidant substances can reduce Ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to produce blue Fe^{2+}-TPTZ, which has a characteristic OD absorbance at 593 nm. The total antioxidant performance was evaluated according to a pre-measured standard curve. According to the kit protocol, the Ferric reducing antioxidant power of the EOF was measured with a total antioxidant capacity (FRAP method) assay kit (JianCheng Bioengineering Institute, Nanjing, China). FRAP working solution was added to each test well of the 96-well plate, following by the addition of EOF at different concentrations. The absorbance was determined at 593 nm after incubation at 37 °C for 3 min.

2.7. In vitro antimicrobial assay

2.7.1. EOF treatment and sample preparation

The bacterial suspensions (7-log CFU/mL) were cultured in different concentrations of EOF in LB (set as the control group, solvent-only group, 0.5 MIC, 1 MIC, and 2 MIC of EOF group) for 0 and 3 h at 37 °C. After incubation, bacterial suspensions were harvested by centrifugation at 6500 rpm for 10 min. The cell pellet was washed twice with fresh LB. Finally, cell suspensions with 7 log CFU/mL concentration were resuspended in LB.

2.7.2. Biofilm formation ability

The biofilm-forming capacity of *E. coli* and *S. aureus* was evaluated using the microtiter-plate-based method described previously, with some modifications (Gajdács et al., 2021). In brief, 100 μL of fresh culture solution was added to each 96-well polystyrene microplate culture plate, inoculated with 10 μL of *E. coli* and *S. aureus* suspension after EOF treatment and cultivated at 37 °C for 24 h. Subsequently, the culture solutions were discarded, and the wells were washed three times using 200 μL of phosphate-buffered saline (PBS; pH at 7.4). 100 μL of 95% methanol was added to each well for 15 min; the liquid was discarded, and the wells were air-dried. Cells were stained with 100 μL 1% (w/v) crystal violet for 10 min, rinsed three times with sterile PBS, and air-dried. 100 μL of 33% (v/v) acetic acid was added to each well. The cells were incubated at 37 °C for 30 min, and the OD value of the solution in the culture well was measured at 630 nm with a microplate reader (iMark, Bio-Rad, CA, USA).

2.7.3. Quantification of viable cells by the cell counting kit-8 (CCK-8) assay

Viable cells were measured with a CCK-8 kit (Beyotime, Shanghai, China). In brief, the bacterial suspension treated with the EOF was diluted to 6-log CFU/mL, and 100 μL was added to each well of a 96-well plate. Then, 10 μL of CCK-8 solution was added to each well, and the plate was incubated at 37 °C for 2 h. CCK-8 metabolic products were measured by a microplate reader with the absorption set at 450 nm. The readings were normalized and expressed as a percentage of surviving bacterial population.

2.8. Antibacterial mechanism

2.8.1. Integrity of the cell wall

Cell wall integrity was evaluated by determining the leakage of alkaline phosphatase (AKP) activity into the bacterial suspension (Yang et al., 2020). After EOF treatment, the AKP activity of the resuspended bacterial suspension was measured with an AKP kit (JianCheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. A microplate reader tested the absorption of supernatants at 490 nm.

2.8.2. Measurement of nucleic acids leakage

The nucleic acid leakage was measured to determine the cell membrane's integrity in the supernatant. 7-log CFU/mL of bacterial suspensions were cultured with different concentrations of the EOF for 0 or 3 h at 37 °C. Then, the suspensions were centrifuged at 8000 rpm for 10 min. The supernatants were collected and filtered through a sterile PTFE microporous membrane of 0.22 μm. The absorbance was measured at 260 nm with a spectrophotometer.

2.8.3. Field emission-scanning electron microscope (FE-SEM) analysis

To observe the morphology of EOF-treated *E. coli* and *S. aureus* cells after 3 h of storage, the treated-bacteria solution was centrifuged (6000×*g*, at 4 °C), and the cells were collected and washed with PBS (pH = 7.4) three times. After washing, the cells were fixed overnight at 4 °C with 2.5% glutaraldehyde PBS buffer solution. After dehydration with ethanol of different concentrations (25, 50, 75, 90, and 100%) for 10 min, the bacteria cells were fixed on silicon wafers, dried in a vacuum oven at 25 °C, and then coated with a gold layer. Finally, the morphology of the *E. coli* and *S. aureus* cells was observed with SEM (Hitachi SU8220, Jeol, Japan).

2.9. Statistical analyses

Relevant experiments were carried out in triplicates. Data were expressed as the means ± standard deviations. Statistical differences were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Analysis was performed using SPSS Statistics software (version 22.0, IBM, Chicago, IL, USA). A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Antibacterial activity assay

The three EO blends were evaluated for their antibacterial activity against specific microorganisms, with the size of the DIZ shown in Fig. S1. The orthogonally optimized EOFs were more active against specific tested strains. As shown in Table S2, the maximum DIZ of *S. aureus* with orthogonal experimental design (OED) of A1B3C3 combination was 18.09 ± 1.08 mm, and the A1B3C3 combination was named EOF1 (containing 5% Tween-80, 10% ethanol, 2% clove oil, 2.5% oregano oil, 1.25% cinnamon oil and water). The maximum DIZ of *E. coli, B. subtilis, S. putrefaciens* and *V. parahaemolyticus* with OED of A2B2C3 combination was 15.11 ± 0.32, 15.75 ± 0.21, 14.20 ± 0.65 and 19.61 ± 0.74 mm, respectively, and the A2B2C3 combination was named EOF2 (containing 5% Tween-80, 10% ethanol, 3% clove oil, 2% oregano oil, 1.25% cinnamon oil and water). The agar disc diffusion method indicated that EOF1 and EOF2 had outstanding antibacterial activity ($p < 0.05$). This could be due to the high contents of cinnamon and oregano oils in both EOF1 and EOF2. Zhang, Liu, Wang, Jiang, and Quek (2016) indicated that cinnamon essential oil exhibited potent antibacterial activity against foodborne spoilage and pathogenic bacteria in model systems using *E. coli* and *Staphylococcus* spp. Clove oil showed the lowest antibacterial activity of the three EOs due to the lowest concentration of antibacterial compounds. Each EO's chemical composition (e.g., cinnamaldehyde, carvacrol, thymol, and eugenol) might explain the antibacterial activity difference (Dogruyol et al., 2020; Liu et al., 2021; Yoo et al., 2021).

Furthermore, the antibacterial activity of EOFs on *S. aureus* and *E. coli* was quantitatively evaluated by the MIC values. As shown in Table S3, EOF1 and EOF2 showed the same antimicrobial activities against the tested strains based on the calculated MIC. The MICs for both EOF1 and EOF2 were 7.940 μL/mL. The results confirmed that EOF composed of three EOs had lower MICs against *E. coli* and *S. aureus*, compared to a single EO from a previous study ($p < 0.05$), confirming its enhanced antibacterial potential (Hu et al., 2021). The higher susceptibility of Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria to EOF was consistent with the results obtained in the vast majority of EO studies (dos Santos Rodrigues et al., 2017; Zhang et al., 2016).

3.2. Physicochemical properties of EOFs

The GC–MS results (Table 1, Fig. S2) show that around 30 substances existed in EOF1 and EOF2. The main ingredients were cinnamaldehyde, carvacrol, eugenol, and certain linalool, consistent with the results reported by Amelia, Saepudin, Cahyana, Rahayu, Sulistyoningrum, and Haib (2017). Dong, He, Xiao, and Li (2020) showed that cinnamaldehyde has a good sustained release effect and can be antibacterial. Cinnamaldehyde could act synergistically with pyrazinamide to fight against pyrazinamide-resistant *Mycobacterium tuberculosis* (Wan, Zhang, Liu, & Yang, 2022). In addition, the synergistic antibacterial ability of carvacrol and eugenol has also been verified in previous articles (Toushik et al., 2022). Carvacrol had a wider range of effects, inducing free radical oxidation of tumor cells, inhibiting fungal mycelia growth, increasing cell membrane permeability, and ultimately causing bacterial component leakage.

The phase inversion emulsification prepared the essential oils emulsion using a surfactant containing Tween-80 and ethanol. EOF1 and EOF2 showed that the O/W emulsion has good water solubility and uniform milky white solutions with no aggregates or visible precipitates (Fig. S3). However, poor water solubility (with separated layers or phases for the EO and water), and the solution was turbid and uneven for the EO mixtures without adding solvents (5% Tween-80, 10% ethanol) for solubilization (Fig. S3). Tween-80 and ethanol were beneficial to the emulsification and dispersion of EO, avoiding the instability of evaluating their antibacterial activity due to uneven dispersion in hydrophilic media. These characteristics still existed after 7 d of storage.

Table 2 shows the droplet size, PDI, zeta potential, pH, and color difference of EOF1 and EOF2. During the 7 d of storage, the PDI of EOF1 and EOF2 was less than 0.3, and at 0 d, the minimum values reached 0.202 and 0.208, respectively. Low PDI (< 0.3) indicated stability and homodispersion of EOFs (Kreutz et al., 2021). Furthermore, the droplet size of EOF1 and EOF2 was 137–146 nm and 137–142 nm, respectively (Fig. S4). The droplet size of EOF1 and EOF2 did not change ($p > 0.05$) during storage, indicating the good stability of the emulsions.

During storage, the corresponding zeta potentials for EOF1 and EOF2 were lower than -14.67 ± 0.06 mV and -15.03 ± 0.31 mV. The high zeta potential values for the emulsions indicated good physical stability due to the high repulsion found (Cui, Lu, Li, Rashed, & Lin, 2022). The negative zeta potentials may be attributed to the adsorption of hydroxyl ions at the O/W interface, the continuous development of hydrogen bonds between these ions, and the oxyethylene moieties of the surfactant Tween-80 (Hong, Kim, & Lee, 2018). In addition, minimal changes in pH (values ~4) and color difference were observed during storage. Notably, the color difference was consistent with the milky white results in Fig. S3, showing a high L* value of about 87 to 88. Previous studies have also found that EOs often present acidic properties in nanoemulsion (<200 nm) systems (da Silva Gündel et al., 2018).

3.3. Antioxidant and antimicrobial activity evaluations of EOFs

DPPH radical scavenging capacity is widely used to study antioxidant activity (Xu, Wei, Jia, & Song, 2020). As shown in Fig. 1**A**, the corresponding DPPH radical scavenging activity for EOF1 and EOF2 was increased and reached 88.71% and 89.63% at a concentration of 15.880 μL/mL. The DPPH radical scavenging activity of EOF1 was higher than that of EOF2 at an EO emulsion concentration of less than 7.940 μL/mL (equivalent to 1 MIC). While further increasing the emulsion concentration, no differences were observed in the DPPH radical scavenging activities between the two emulsions. The FRAP of EOFs is displayed in Fig. 1B. Compared to DPPH scavenging, the FRAP of EOF2 was higher

Table 1
EOs emulsion composition from internal normalization of GC–MS chromatogram.

Peak	RT (min)	*m/z*	Formula	Compounds	CAS	Percentage* %	
						EOF1	EOF2
1	4.583	45	C2H6O	Ethyl alcohol	64-17-5	23.67	23.32
2	14.492	93	C10H16	α-pinene	7785-70-8	0.46	0.45
3	14.873	121	C10H16	Camphene	79-92-5	0.13	0.13
4	15.435	93	C10H16	β-pinene	18172-67-3	0.15	0.15
5	15.496	69	C10H16	β-Myrcene	123-35-3	0.35	0.29
6	15.932	93	C10H16	α-Phellandrene	99-83-2	0.19	0.18
7	16.124	121	C10H16	α-terpinene	99-86-5	0.48	0.41
8	16.264	119	C10H14	o-Cymene	527-84-4	2.91	2.46
9	16.359	68	C10H16	D-Limonene	5989-27-5	0.45	0.46
10	16.422	93	C10H16	Sabinene	3387-41-5	0.24	0.22
11	16.858	93	C10H16	γ-Terpinene	99-85-4	1.5	1.23
12	17.356	32	C10H16	α-terpinene	99-86-5	0.09	0.08
13	17.49	93	C10H18O	Linalool	78-70-6	3.13	2.71
14	18.911	95	C10H18O	*endo*-Borneol	507-70-0	0.31	0.24
15	18.99	71	C10H18O	4-terpineol	20126-76-5	0.55	0.45
16	19.187	59	C10H18O	L-α-Terpineol	10482-56-1	0.24	0.22
17	19.888	32	C9H10O	Chavicol	501-92-8	—	0.07
18	20.409	131	C9H8O	E-Cinnamaldehyde	14371-10-9	15.43	14.72
19	20.614	135	C10H14O	Carvacrol	499-75-2	18.94	16.5
20	21.461	164	C10H12O2	Eugenol	97-53-0	18.37	21.29
21	21.547	135	C12H16O2	Carvacryl acetate	6380-28-5	0.26	—
22	21.902	119	C15H24	Copaene	3856-25-5	0.18	0.23
23	22.49	93	C15H24	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	242794-76-9	4.98	5.87
24	22.544	32	C11H12O2	(E)-Cinnamyl alcohol acetate	21040-45-9	0.08	—
25	22.689	93	C15H24	Aromandendrene	489-39-4	0.37	0.08
26	22.874	164.1	C15H24	1,4,7-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	1000062-61-9	5.69	0.49
27	23.218	161.1	C12H14O3	Acetyl eugenol	93-28-7	0.14	6.83
28	23.384	69	C15H24	γ-cadinene	39029-41-9	0.36	—
29	23.383	161.1	C15H24	δ-cadinene	483-76-1	—	0.15
30	23.450	159.1	C15H22	*cis*-Calamenene	72937-55-4	—	0.13
31	24.085	44	C15H24O	Caryophyllene oxide	1139-30-6	0.1	0.41
32	25.31	104.9	C14H12O2	Benzyl benzoate	120-51-4	0.12	0.14

* The relative peak area of the specific compound (%) to the whole.

Table 2
Physicochemical characterization of essential oil emulsion.[1]

	EOF1		EOF2	
	Day 0	Day 7	Day 0	Day 7
Droplet size (nm)	137.6 ± 0.9 a	145.7 ± 5.9 a	137.1 ± 1.4 a	142.0 ± 3.4 a
Polydispersity index	0.202 ± 0.012 a	0.207 ± 0.013 a	0.208 ± 0.003 a	0.209 ± 0.008 a
Zeta Potential (mV)	−18.00 ± 0.90 a	−14.67 ± 0.06b	−16.07 ± 1.15 a	−15.03 ± 0.31 a
pH	4.06 ± 0.02 a	4.04 ± 0.03 a	4.03 ± 0.01 a	3.99 ± 0.03 a
L*	87.36 ± 0.61 a	87.33 ± 0.17 a	88.62 ± 0.25 a	88.15 ± 0.21b
a*	−3.65 ± 0.76 a	−2.23 ± 0.30b	−1.89 ± 0.31 a	−1.79 ± 0.18 a
b*	−0.33 ± 0.12 a	1.13 ± 0.18b	0.75 ± 0.12 a	1.46 ± 0.20b

[1] *Values were expressed as mean ± standard deviation (n = 3). A T-test was conducted between two different time groups of the same essential oil sample, and different lowercase letters indicate significant differences (p < 0.05).*

than that of EOF1 at the initial EOs emulsion concentration. When the concentration of the EOFs reached 3.750 μL/mL, the observed FRAP differences decreased and remained stable. More specifically, the FRAP of EOF1 and EOF2 increased with EOs emulsion concentration and reached 1.802 and 1.797 mM $FeSO_4{\cdot}7H_2O$, respectively, at 15.880 μL/mL.

Although DPPH and FRAP of the two EOFs exhibited opposite trends at the initial concentration, the differences decreased with increasing EO emulsion concentrations, which could be attributed to the poor stability of the emulsions at low concentrations. Briefly, the composite EO emulsions could effectively remove free radicals and showed high reducing power. Huang, Liu, Zhang, and Guan (2021) demonstrated that an oil-in-water cedarwood EO emulsion had good solubility in an aqueous solution, compared to the pure EO, and was suitable for rapidly transporting active components to the solution.

3.4. *In vitro antimicrobial assay*

Biofilms are complex communities in which foodborne bacteria adhere to food surfaces. Microorganisms in biofilms are more resistant than planktonic microorganisms during sterilization treatment, seriously threatening food safety (Cui, Bai, Sun, Abdel-Samie, & Lin, 2018). This study analyzed the effect of EOFs on the biofilm formation ability of *S. aureus* and *E. coli*. The crystal violet assay showed that EOFs could inhibit the biofilm formation of *S. aureus* (Fig. 2A) and *E. coli* (Fig. 2B). The biomass of *S. aureus* decreased by 11.19%, 15.32%, and 18.09%, and that of *E. coli* decreased by 14.25%, 21.72%, and 22.37%, after exposure (0 h) to EOFs at 0.5 MIC, 1 MIC, and 2 MIC, respectively. With the EOFs treatment time extended to 3 h, the biofilm decreased in the 1 and 2 MIC groups was approximately doubled. These results demonstrated that the biofilm formation of *S. aureus* and *E. coli* was sensitive to EOFs, and EOFs were highly influential in restricting bacterial biofilm growth.

In addition, Fig. 2C and Fig. 2D showed that the cell viability of *S. aureus* and *E. coli* decreased when incubated with higher concentrations of EOFs. The CCK-8 assay also confirmed this concentration-responsive effect. Compared to the control and solvent-only (SO) groups, *S. aureus* and *E. coli* cell viability in the EOF-treated groups decreased significantly ($p < 0.05$). However, the reduction in cell viability did not exceed 50% until the 1 MIC concentration of EOFs was added. Thus, bacterial cell viability decreased depending on EOF content and treatment time. These results demonstrated that the EOFs

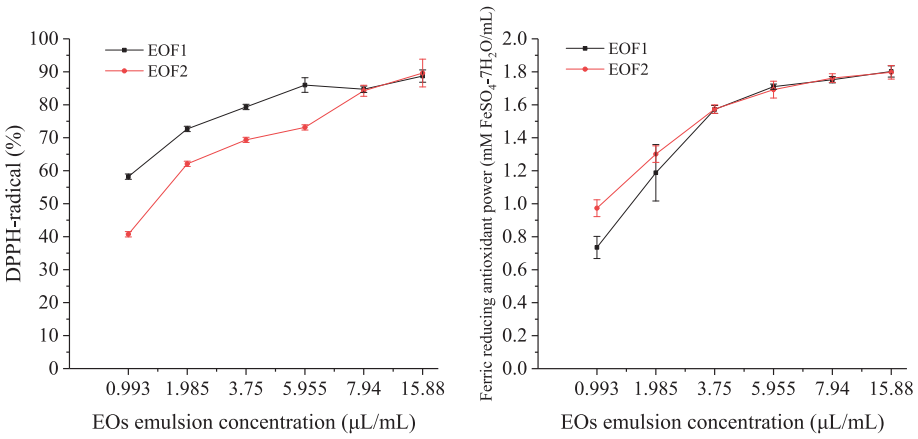

Fig. 1. Effects of antioxidant properties of EOs emulsion under different concentrations. (A) DPPH radical scavenging ability of EOs emulsion; (B) Ferric reducing antioxidant power of EOs emulsion. EOs: essential oils. Error bars denote mean (n = 3) ± SD.

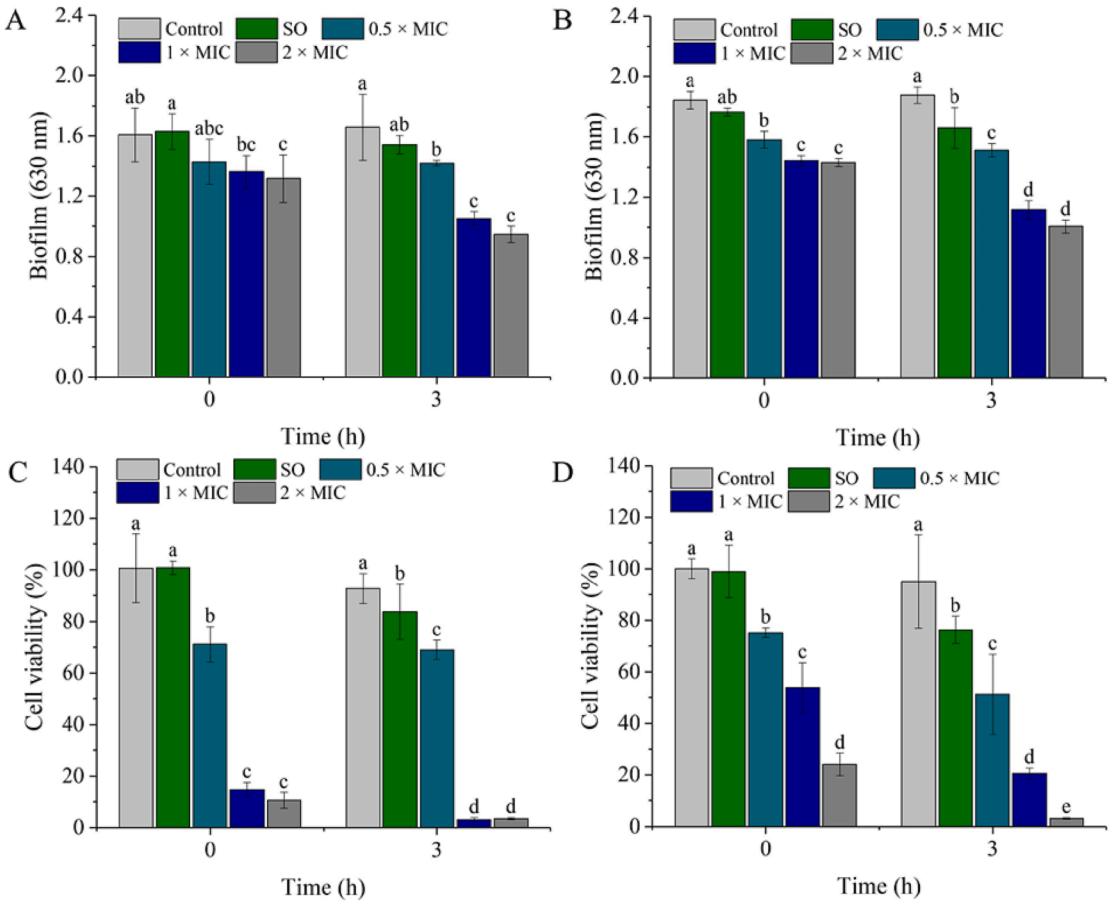

Fig. 2. Effects of EOs emulsion on biofilm formation and cell viability of *S. aureus* and *E. coli* at 37 °C. (A) Biofilm formation of *S. aureus*; (B) Biofilm formation of *E. coli*; (C) Cell viability of *S. aureus*; (D) Cell viability of *E. coli*. SO: solvent-only group; EOF1: essential oil formula group 1; EOF2: essential oil formula group 2; EOs: essential oils. Error bars denote mean (n = 3) ± SD; Different lowercase letters indicated a significant difference between EOs emulsion concentrations for the same strain, $p < 0.05$.

possess potent antibacterial activity against *S. aureus* and *E. coli*. The possible antibacterial mechanism of EOFs against *S. aureus* and *E. coli* was further investigated in the next section.

3.5. Antibacterial mechanism

Due to the diversity of EOs chemical components, microbial cells have no specific mechanisms of action. However, the most frequently discussed inactivation mechanism involves increased cell membrane permeability. Moreover, the lipophilicity of EOs promotes the targeted diffusion and interaction of chemically active components to the cell membranes and intracellular components. In addition, the increase in cell membrane permeability also led to the leakage of cytoplasmic content, causing cell death (Cui et al., 2018; Guo et al., 2019).

AKP is an enzyme found between the bacterial cell wall and cell membrane. Therefore, bacterial cell wall integrity results can be

obtained by measuring the AKP activity leaked from cells (Yang et al., 2020). The higher activity of the AKP, the more severe the observed cell wall effects. As shown in Fig. 3A & B, the AKP activity increased obviously in all samples treated with EOFs, especially for those with higher EOF concentrations (2 MIC). These results indicate that EOF treatment can instantaneously change the cells' permeability and integrity, leading to the leakage of AKP from bacterial cells. In addition, it was found that EOFs lead to different leakage levels of AKP in Gram-positive and Gram-negative bacteria, with the former higher. This may be due to cell wall composition differences (Zhang, Lan, Wang, Sun, & Xie, 2018).

The cell membrane is an important protective barrier for bacteria and is essential to cell functions. Even small changes in bacterial cell membrane integrity can lead to cell death (Cox et al., 2001; Kang et al., 2019). The release of intracellular components (nucleic acids) from the damaged cell membrane after EOFs treatment was quantitatively evaluated by measuring the absorbance at 260 nm, and the results are shown in Fig. 3C & D. At the initial stage of EOFs treatment, the nucleic acid leakage level in the treatment groups was not sensitive to the change in EOFs concentrations. Nonetheless, as the storage time was extended to 3 h, nucleic acid leakage in OD at 260 nm increased significantly ($p < 0.05$) with the increased EOFs concentration and prolonged storage time. Additionally, the nucleic acid leakage of *S. aureus* (Fig. 3C) was higher than that of *E. coli* (Fig. 3D), consistent with the results of the AKP activity. These findings suggested that the *S. aureus* and *E. coli* cell membrane integrity was destroyed after EOFs treatment, leading to increased extracellular nucleic acids in the treated cells.

The SEM micrographs of *S. aureus* and *E. coli* in control, SO, and 1 MIC treated groups are shown in Fig. 4. Before EOFs treatment, *S. aureus* (Fig. 4A, 4D) and *E. coli* (Fig. 4G, 4J) cells had intact cell structure without damage to the cell wall or cell membrane. There was no cytoplasm leakage, and the cells exhibited a smooth cell surface in the control group. Concomitantly, for the SO treatment group, there was no significant change in *S. aureus* (Fig. 4B, 4E), and the cells remained intact and smooth. Similarly, *E. coli* exhibited no obvious changes except for some bacterial surface folds (Fig. 4H, 4K). Conversely, after 1 MIC EOFs treatment, the morphological damage and intracellular leakage of some *S. aureus* cells were observed (Fig. 4C, 4F). In contrast, the morphology of *E. coli* cells was seriously damaged, with almost no complete morphology observed (Fig. 4I, 4L). Zhang et al. (2016) reported the inhibitory effect of commercial cinnamon EO on *S. aureus* and *E. coli,* with changes to the cell wall and cell membrane structure observed. The mechanism directly led to a decrease in cell viability. In this study, the cell membrane damage to *S. aureus* and *E. coli* was observed in the EOFs treatment groups, which supported the antibacterial mechanism of the EOFs tested.

4. Conclusion

In this work, an emulsion containing composite ordinary EOs with enhanced water solubility was prepared by the phase inversion method. This property enabled full contact of EO emulsions with the hydrophilic substrate, improving their antibacterial activity against exogenous

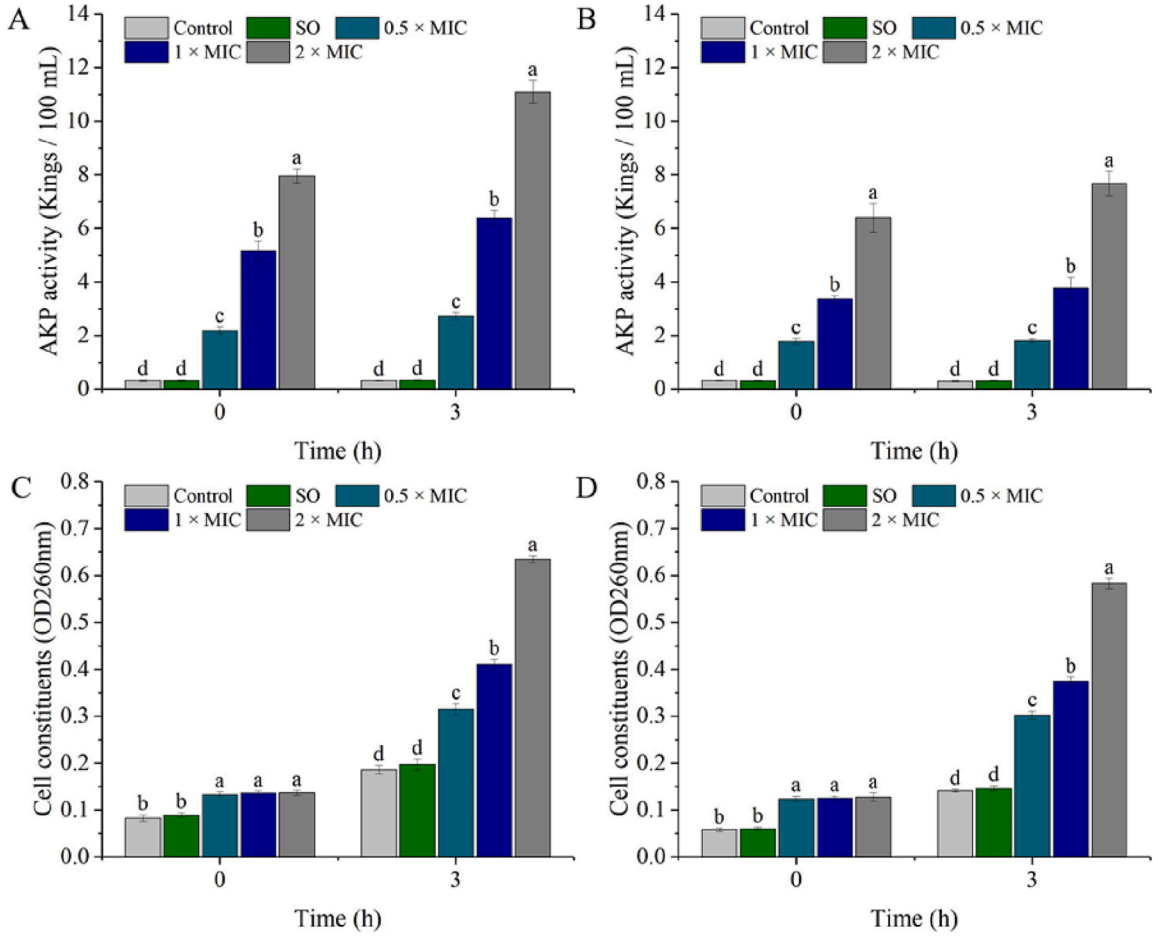

Fig. 3. Effects of EOs emulsion on the cell wall and cell membrane of *S. aureus* and *E. coli*. (A) AKP activity of *S. aureus* and (B) *E. coli*; (C) Measurements at leaking intracellular compoents of *S. aureus, and* (D) *E. coli*. SO: solvent-only group; EOF1: essential oil formula group 1; EOF2: essential oil formula group 2; EOs: essential oils; Error bars denote mean (n = 3) ± SD; Different lowercase letters indicated a significant difference between EOs emulsion concentrations for the same strain, $p < 0.05$.

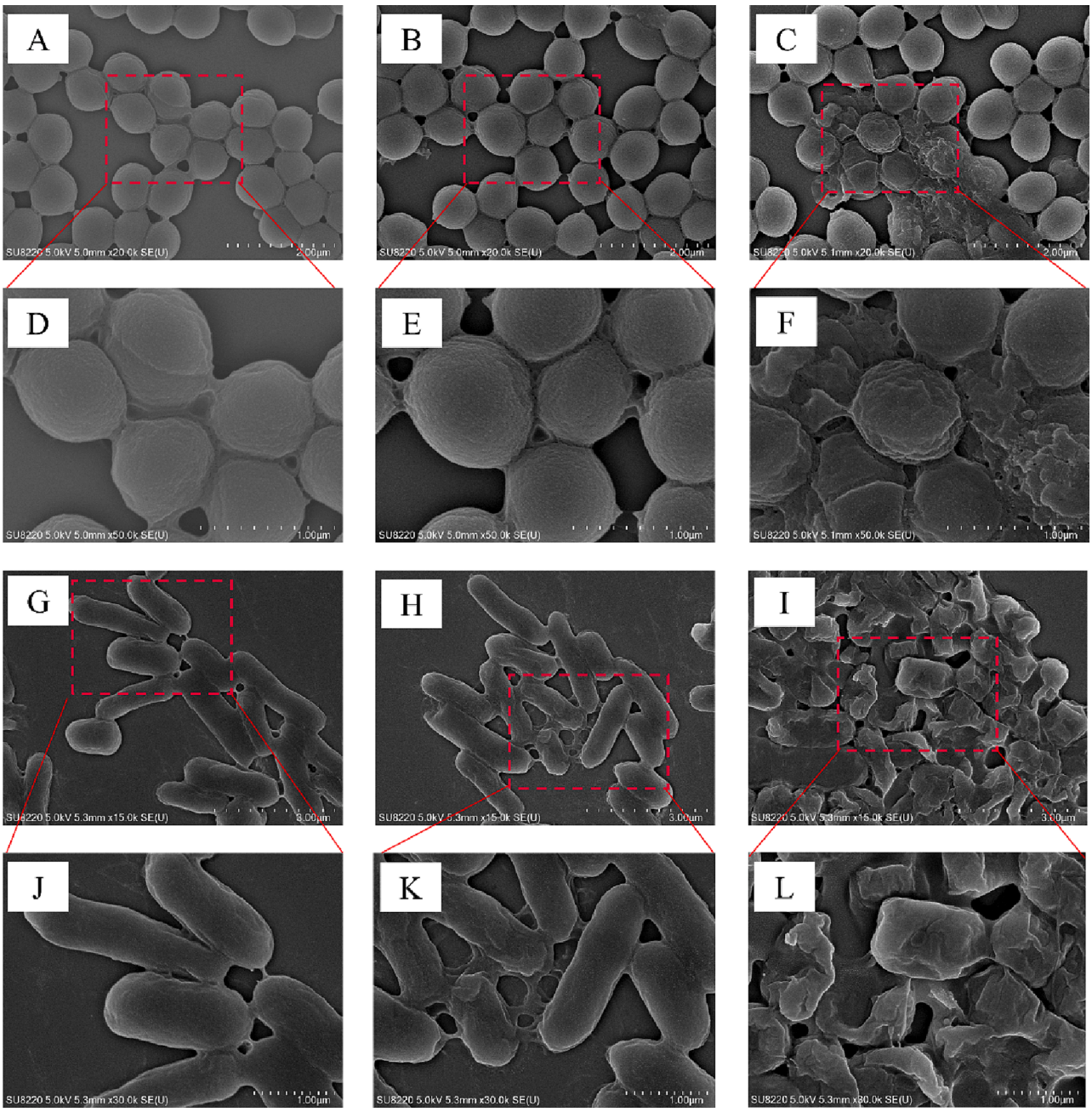

Fig. 4. SEM images of *S. aureus* (× 20 k, A-C, and × 50 k, D-F), without EO emulsions (A, D), treated with SO (B, E) and 1 × MIC EOF (C, F), and *E. coli* (×15 k, G-I, and 30 k, J-L) without EOs emulsion (G, J) treated with SO (H, K) and 1 × MIC EOF (I, L).

microorganisms. The EO emulsions were rich in cinnamaldehyde, carvacrol and eugenol, which are identified for their high antioxidant and antibacterial properties. The composite EOFs prepared based on the orthogonal optimization have reliable post-storage stability (over 7 days) and broad-spectrum antibacterial benefits. The antibacterial studies of EOFs against *S. aureus* and *E. coli* showed that cell surface and dehydroreductase were the attack targets. Loss of cell wall and cell membrane integrity, leakage of intracellular substances and retardation of the respiratory chain were the main reasons for the bacterial inactivation and anti-biofilm formation, featuring their potential in food applications.

CRediT authorship contribution statement

Jiajie Hu: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. **Hangxin Zhu:** Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Yuwei Feng:** Validation, Writing – original draft. **Mijia Yu:** Investigation. **Yueqiang Xu:** Investigation. **Yadong Zhao:** Resources, Writing – review & editing. **Bin Zheng:** Resources, Funding acquisition. **Jiheng Lin:** Resources, Writing – review & editing. **Wenhua Miao:** Conceptualization, Methodology, Supervision, Funding acquisition, Writing – review & editing. **Rusen Zhou:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Supervision. **Patrick J. Cullen:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136201.

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