Synergistic antibacterial effects of carvacrol and ε-polylysine

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ORIGINAL RESEARCH

Abstract

This study aimed to evaluate the antimicrobial efficacy of the combination of ε-polylysine (ε-PL) and carvacrol (Car) against foodborne pathogens, *Escherichia coli* and *Staphylococcus aureus*. The minimum inhibitory concentrations (MICs) of ε-PL (Car) against *E. coli* and *S. aureus* were 25 μg/mL (320 μg/mL) and 12.5 μg/mL (320 μg/mL), respectively. Checkerboard assays showed that the combination of ε-PL and Car exerted synergistic effects against *E. coli* and *S. aureus* with fraction inhibitory concentration index (FICI) of 0.375 and 0.5, respectively. It demonstrated that the combination of ε-PL and Car significantly inhibited the growth of the two strains compared to single treatment. Furthermore, the mode of action of ε-PL (6.25 μg/mL) or Car (80 μg/mL) in inhibiting *E. coli* and *S. aureus* was researched by assessing their changes with regard to cellular membrane integrity, membrane permeability, respiratory activity, and membrane structure. A combination of ε-PL and Car increased the damage to cell membranes and their permeability and led to the release of 260 nm absorbing materials, decreased respiratory-chain dehydrogenase activity compared with ε-PL or Car treatment alone. These results demonstrated that the combination of ε-PL and Car could be used as a new promising naturally sourced food preservative.

Keywords: carvacrol; ε-polylysine; synergistic antimicrobial activity

Introduction

The Centres for Disease Control (CDC) estimates that each year approximately one in six Americans (or 48 million people) fall sick, of which 128,000 are hospitalized, and 3000 die of foodborne diseases. Therefore, food safety issues caused by foodborne diseases have received widespread attention. Pathogens causing the most foodborne illnesses, hospitalizations, and deaths each year include *Escherichia coli* O157:H7, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Campylobacter*. In particular, recent studies have reported that meat contaminated with pathogenic bacteria, such as *E. coli* O157:H7, *L. monocytogenes*, and *Bacillus cereus*, represents a serious public health risk. Some *E. coli* strains are commensal both for human and animal intestines, and Shiga-toxin producing strains are responsible of food-related infections (Pinilla and Brandelli, 2016). Awareness of synthetic additives and preservatives in food processing has led food industries to search for natural additives with a broad spectrum of antimicrobial activities. Food consumers and enterprises urgently need natural alternatives to assure food safety and quality (Saharkhiz *et al*., 2016).

Essential oils are volatile compounds from plant materials that have been used as naturally derived antimicrobials for food bio-preservatives (Vergis *et al*., 2015).

Among the different groups of chemical constituents of essential oils, one of the most effective is carvacrol (Car for short) (Lambert *et al*., 2001). It has been reported that the essential oils of the oregano and thyme were effective against strains of *E. coli* (Dorman and Deans, 2010). The major antibacterial component of these oils is Car, which effectively inhibits the growth and survival of several foodborne pathogens, such as *S. aureus* and different strains of *E. coli*. Car exhibits antibacterial activity against the foodborne pathogen *B. cereus* by disrupting the cell membrane. Car reduces intracellular adenosine triphosphatase ATP and membrane potential, leading to dissipation of the pH gradient and cell death (Ultee *et al*., 1999). Car is hydrophobic and disrupts the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP (Lambert *et al*., 2001). Numerous lines of evidence indicate that Car's site of action is the membrane and that Car's mode of action is to increase membrane fluidity and permeability. Car is naturally occurring, but the main obstacle to the use of Car as a food preservative is that it is often not potent as a single compound. Car causes negative organoleptic effects, including a strong flavor and odor, when used at levels necessary to provide antimicrobial effects (Lambert *et al*., 2001). Many studies suggest that Car is synergistic with other antimicrobial preservatives. Thus, the amount of Car applied to foods could be reduced and the unpleasant effects of high doses of Car could be avoided (Palaniappan and Holley, 2010).

ε-Poly-L-lysine (ε-PL for short) is a homopolyamino acid characterized by a peptide bond between the carboxyl and α-amino groups of l-lysine. This molecule is composed of a series of 25–35 L-lysine monomers and has a molecular weight of approximately 5000 Da (Sun *et al*., 2018). ε-PL is derived from metabolites produced by the fermentation of *Streptomyces albulus* (Li *et al*., 2014) and is a highly safe natural food preservative. Compared with chemical preservatives, ε-PL is a safer product of biological fermentation that can be broken down into lysine required by the human body (Zahi *et al*., 2017). In addition, ε-PL also has the advantages of a broad antibacterial spectrum, high temperature resistance, good water solubility, low toxicity, and efficacy in a wide range of pH values. Regarding its mode of action against *E. coli*, ε-PL reduces the amounts of large molecules, soluble cellular proteins, and nucleic acids by damaging cell membranes (Zhang *et al*., 2018). In addition, ε-PL is believed to electrostatically adsorb onto the cellular membrane, resulting in membrane disruption and abnormal distribution of the cytoplasm (Shima *et al*., 1984). In addition, ε-PL has been approved as a safe food preservative in China and the United States and has been widely used in the preservation of various foods, such as cooked meat products, fruit and vegetable juices, and egg products. However, ε-PL can be subject to rapid depletion after initial application and lose activity quickly (Bi *et al*., 2016). Studies have been performed to improve the effectiveness of ε-PL. For example, Lin *et al*. (2018) confirmed that the gelatine nanofibers containing thyme essential oil/beta-cyclodextrin epsilon-polylysine nanoparticles were engineered in order to control the propagation of *C. jejuni*. ε-PL or ε-PL combined with CO_2 packaging is effective in controlling the food-borne pathogens, and its covalent immobilization to multi-walled carbon nanotubes is useful in the construction of a nanocomposite with enhanced antibacterial activity (Miya *et al*., 2016).

In order to improve the antimicrobial activity, a novel combination of antimicrobial agents is extensively used. According to reports, the combination of EOs and other EOs or natural antibacterial agents can achieve a strong inhibitory effect of very low doses and effectively reduce the negatives (Govaris *et al*., 2010). On the other hand, the antibacterial effect and mechanism of action of the combination of ε-PL and Car have not been investigated to date. The objectives of this study were to investigate the synergistic action of the combined substances (ε-PL and Car) against *E. coli* and *S. aureus* using *in vitro* assays and verify the application feasibility of this combination as a promising new antibacterial agent in food preservation. *E. coli* was chosen as a model for pathogenic Gram-negative bacteria (Sondi and Salopek-Sondi, 2004), whereas *S. aureus* was selected as a representative strain of Gram-positive bacteria. Both bacteria served as representatives for foodborne diseases. Therefore, our study seeks to elucidate the synergistic bacteriostatic effect of ε-PL and Car, and preliminarily investigate their antibacterial mechanism.

Materials and Methods

Chemical reagents and bacterial strains

Carvacrol and ε-polylysine were purchased from Shanghai McLean Corporation (Shanghai, China). Iodonitrotetrazolium blue (INT) was obtained from Shanghai Sobo Biotechnology Co., Ltd. (Shanghai, China). Sodium chloride, tryptone, and yeast extract were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). In addition, 12- and 24-well cell culture plates were obtained from Corning Incorporated (New York, USA). The BCA protein quantification kit was purchased from Biotech Bioengineering Co., Ltd (Shanghai, China). Two food-related microorganisms were used to assess antimicrobial properties, including Gram-negative *E. coli* CICC 10664 and Gram-positive *S. aureus.* CICC 21600. The two strains were obtained from the China Centre of Industrial Culture Collection, CICC (Shanghai, China). Luria-Bertani Broth (LB) (1%

tryptone, 0.5% yeast extract, and 1% sodium chloride) was used for the growth of bacterial cultures (Shanghai Jingan Biotechnology Co., Ltd, Shanghai, China). The remaining reagents are of analytical grade.

Determination of MIC and MBC

The minimal inhibitory concentrations (MICs) of ε-PL and Car against bacteria were determined based on Clinical and Laboratory Standards Institute guidelines using the standard broth dilution method as previously described (Chen and Zhong, 2017). First, 100 μL of LB liquid culture medium was added to each well of the 96-well plate. Then, 100 μL of bacteriostatic agent was added to the first well of each row and mixed thoroughly. Transfer 100 μL of the above solution from the first well to the second well, then transfer 100 μL from the second well to the third well and repeat until it is added to the 11th well in the same row. The solution to well 11 was mixed, and 100 μL was removed. Then, 100 μL of *S. aureus* or *E. coli* (~10^7 CFU/mL) diluted with physiological saline was added to wells 1 to 10 and 12 of each row, and 100 μL of physiological saline was added to well 11. The above sample was incubated at 37°C for 24 h in a constant temperature incubator. $OD_{600\ nm}$ value of each well was detected using SpectraMax iD3 multifunctional microplate reader. The lowest concentration of Car and ε-PL that inhibits the growth of *E. coli* or *S. aureus* in the medium is their MIC.

Refer to the method of Chen *et al*. (2017) with a slight modification. According to the results of the abovementioned minimum inhibitory concentration (MIC), for negative wells, a 100 μL aliquot was spread on the LB medium, and then incubated at 37°C for 48 h. The concentration of aseptic growth was determined as MBC.

Checkerboard microdilution tests

The synergistic interaction between Car and ε-PL was determined using the checkerboard test based on a previous study (Kozak *et al*., 2018). Serial dilutions of both antimicrobials were mixed to obtain a fixed amount of the first agent and increasing amounts to the second agent in each row (or column). Continuous twofold dilutions of Car and ε-PL were mixed in LB medium. The final concentration of the mixture was 1/32 to 4 times the MIC of Car and 1/128 to 4 times the MIC of ε-PL. The inoculum of each well was adjusted to a final concentration of 1 × 10^7 CFU/mL, and the plate was incubated at 37°C for 24 h.

In order to evaluate the antibacterial effect of each combination, the following equation was used to analyze the data generated by the checkerboard test according to the Fractional Inhibitory Concentration Index (FICI).

Among them, MIC_A and MIC_B are the MIC of the compounds Car and ε-pL when acting alone, and C_A^{COMB} and C_B^{COMB} are the MICs of Car and ε-pL in combination. The interaction between A and B was interpreted as a synergistic effect (FICI ≤ 0.5), partial synergism (0.5 < FICI < 1.0), no effect (1.0 ≤ FICI ≤ 4.0), or antagonism (FICI > 4.0) (Oliveira *et al*., 2010).

Growth kinetic tests

The growth kinetic test was used to evaluate the synergistic antimicrobial effect of Car and ε-PL against *E. coli* or *S. aureus* (Chen *et al*., 2017). Connect a test tube containing 9 mL of sterile LB medium to 2% freshly cultured *S. aureus* or *E. coli* bacterial solution, and add 1 mL sample to the test tube with a final concentration of 80 μg/mL Car or/and 6.25 μg/mL ε-PL. Place it in a constant temperature shaker at 37°C for shaking culture, take samples every 2 h, and measure the absorbance at OD_{600nm}. The growth curve of *E. coli* or *S. aureus* was established according to the correlation between the incubation time and the OD_{600nm} value.

Measurement of the release of 260 nm absorbing cellular materials

The release of UV-absorbing materials was measured using an UV-7504c spectrophotometer according to the method described by Windiasti *et al*. (2019) and Shi *et al*. (2017) with some modifications. Bacteria cells cultured overnight in the LB medium were collected by centrifuge at 5000 g for 15 min at 4°C. The pellets were thoroughly rinsed with phosphate buffer (PBS, pH 7.4) and resuspended in phosphate buffer (PBS, pH 7.4). The absorbance of the final cell suspension at 420 nm was adjusted to 0.7. The above samples were treated with Car (80 μg/mL), ε-PL (6.25 μg/mL), and Car (80 μg/mL)/ε-PL (6.25 μg/mL), respectively. No antibacterial substance added cell suspensions served as negative controls. Bacterial suspensions were incubated at 37°C. The $OD_{260\ nm}$ was determined at 0, 1.0, 2.0, 3.0, 4.0, and 5.0 h after incubation. After incubation, 5.0 mL of each sample was collected at each time-point and centrifuged (11,000 × *g*, 10 min), and the supernatants were filtered through a sterile nitrate cellulose membrane (0.22 μm, Tianjin Jinteng Technology Co., Ltd.). The $OD_{260\ nm}$ value of the supernatant was measured to observe the amount of extracellular UV-absorbing materials released by cells. All the measurements were performed in triplicate.

Membrane permeability assay

Propidium iodide (PI) staining was used to assess membrane integrity of *S. aureus* or *E. coli*. Log phase *S. aureus* or *E. coli* cells (~10^7 CFU/mL) was treated by 80 μg/mL Car and 6.25 μg/mL ε-PL alone or by a combination of 80 μg/mL Car and 6.25 μg/mL ε-PL, and the sample without treatment was used as the control group. After treatment, bacteria cells were harvested by centrifugation at 8000 *g* for 10 min. Then, PI was added to each group and incubated in the dark for 30 min. The membrane permeability of PI-stained cells and the FL3 (red) fluorescence channel was detected under photoexcitation at a wavelength of 488 nm and 615 nm, respectively, by using the FACS LSRFortessa flow cytometry (Becton, Dickinson and Company, America) (Sun *et al*., 2017). The data were analyzed by CellQuest Pro software.

Respiratory chain dehydrogenase determination

E. coli or *S. aureus* was inoculated into the LB medium and incubated at 37°C for 18 h. The bacterial cells were collected by centrifuge for 15 min and washed with physiological saline. Then, the above pellet was resuspended by 1 mL of 1×MIC Car or ε-PL and incubated at room temperature for 3 h.

The bacteria were killed by a water bath at 100°C for 15 minutes to destroy the respiratory chain dehydrogenase activity on the surface of the bacteria. A blank control of live bacteria (without inhibitor) and a negative control of dead bacteria were also prepared.. The sample was centrifuged at 4°C for 15 min again and the pellet was collected, washed, and resuspended in 0.9 mL PBS. Then, 0.1 mL of the respiratory chain dehydrogenase substrate, namely, 0.5% iodine nitro tetrazolium chloride (INT), was added to the above samples. The solution was mixed well and incubated at 37°C for 1 h in the dark. A490 nm values of the reactants were measured using a SpectraMax iD3 microplate reader (Meigu Molecular Instruments (Shanghai) Co., Ltd., China) (Cui *et al*., 2018).

SEM assay

After treatment for ε-PL, Car, or a combination of ε-PL and Car, morphological changes of *E. coli* or *S. aureus* were observed using Scanning Electronic Microscopy. Fresh cultures of *S. aureus* or *E. coli* were diluted with LB medium to 10^7 CFU/mL. Then, 1 mL of the diluted bacterial solution was added to the 24-well plate. A sterile circular slide was placed into each well, and the plate was incubated at 37°C for 12 h. Then, the bacterial suspension was aspirated and washed thrice with sterile PBS. For the experimental groups, 1 mL ε-PL (6.25 μg/mL) and 1 mL Car (80 μg/mL) alone or in combination were added to each well of a 96-well plate. In addition, 1 mL LB liquid medium was added as a control. After incubating at 37°C for 6 h, the slide was removed, washed thrice with PBS, and fixed overnight with 2.5% glutaraldehyde solution to room temperature. Then, the slide was washed with PBS and subject to gradient dehydration using ethanol (30, 50, 60, 70, 80, 90, 95, and 100%; each treatment is 15 min). Then, the slide is soaked in absolute ethanol containing anhydrous sodium sulfate, dried, and sprayed with gold. GeminiSEM 300 field emission scanning electron microscopy is used to observe the cells. The data represent three experiments with similar results (Khlaifat *et al*., 2019).

Statistical analysis

All tests were conducted in triplicate. Data were expressed as the mean ± standard errors. Statistical analyses were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA), and significant differences were reported with a 95% confidence interval ($P < 0.05$).

Results

MIC and FICI of Car and ε-PL against tested bacteria

The growth of *S. aureus* or *E. coli* was effectively inhibited by Car with an MIC of 320 μg/mL. Moreover, their growth was also effectively inhibited by ε-PL with MICs of 12.5 μg/mL and 25 μg/mL, respectively (Table 1). The synergistic bacteriostatic effect of Car and ε-PL was evaluated because two bacteriostatic agents with different bacteriostatic mechanisms may produce a synergistic effect. Specifically, 80 μg/mL Car (1/4 MIC) and 6.25 μg/mL ε-PL (1/2 MIC) exhibited synergistic antibacterial activity against *S. aureus* and *E. coli*, and their FICI values are 0.5 and 0.375, respectively.

In this research, Car and ε-PL alone or in combination showed different antibacterial activities against the tested strains based on the MIC values as shown in Table 1. Synergistic effects between Car and ε-PL were measured by broth dilution checkerboard assay. The bacteriostatic concentration indexes value of Car + ε-PL against *S. aureus* was 0.5, which indicates that they have a synergistic effect. The combined antibacterial effect of Car + ε-PL on *S. aureus* is two times higher than that of any single agent. In addition, Car + ε-PL's bacteriostatic concentration indexes value of *E. coli* is 0.375, which also indicates the synergistic effect on Car and ε-PL. The combined bacteriostatic activity of Car and ε-PL for *E. coli* was increased two and four times, compared with the single components. In conclusion, the best antibacterial activity

Table 1. The inhibitory activity of Carvacrol or/and ε-Polylysine against *S. aureus* or *E. coli*.

Strains	MIC (μg/mL)		FIC (μg/mL)		FICI	Interpretation
	Car	ε-PL	Car	ε-PL		
S. aureus	320	12.5	80	6.25	0.5	Synergism
E. coli	320	25	80	6.25	0.375	Synergism

MIC, Minimal inhibitory concentration; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index.

was obtained using a combination of ε-PL and Car with FIC index values of 0.375 and 0.5, respectively, yielding a synergistic effect on the tested microorganisms. This study demonstrated that ε-PL and Car in combination effectively inhibited *S. aureus* and *E. coli* growth based on FICI values.

Growth curve determination

To further prove the synergistic antimicrobial effect of the two reagents, growth dynamics of *E. coli* and *S. aureus* in Luria-Bertani culture medium as measured by $OD_{600\ nm}$, as shown in Figure 1. When 80 μg/mL Car and 6.25 μg/mL ε-PL were applied individually, the growth curves followed the same trend as the control. In contrast, the growth of *E. coli* and *S. aureus* was completely prevented by the combination of Car and ε-PL during the 24 h incubation with no noticeable change in $OD_{600\ nm}$. The results further confirmed that Car and ε-PL exhibit a synergistic antibacterial effect against *E. coli* and *S. aureus.*

Release of the release of 260-nm absorbing cellular materials

Cytoplasmic membrane permeability was determined based on UV-absorbing release materials, as presented in Figure 2. The $OD_{260\ nm}$ value of the group treatment for Car + ε-PL is higher than that of either alone, which indicated that more 260-nm absorbing materials (these materials are assumed to be primarily DNA, RNA, and metabolites (Teethaisong *et al.*, 2014)) was released outside the cells. When *E. coli* was exposed to Car or ε-PL alone, the release of cellular components increased significantly with time, and the OD values increased to 0.432 and 0.304 at 5 h, respectively. Compared with other groups, the concentration of cell components in the suspension treated with Car combined with ε-PL at OD 260 nm increased to 0.591 at 5 h. When *S. aureus* was exposed to Car or ε-PL alone, the release of cell components increased significantly with time, and the OD values increased to 0.351 and 0.317 at 5 h. However, compared with other groups, the concentration of cell components in the suspension treated with Car combined with ε-PL at OD260 nm increased to 0.548 at 5 h. This result suggests that the cytoplasmic membrane permeability was increased when treated with Car and ε-PL in combination and also illustrates the synergistic effect between them.

Membrane permeability analysis

Changes in the integrity of the cytoplasmic membrane of *E. coli* and *S. aureus* cells were assessed by propidium iodide (PI), a membrane-impermeant dye, via flow cytometric analysis. PI is a nuclear staining reagent that stains

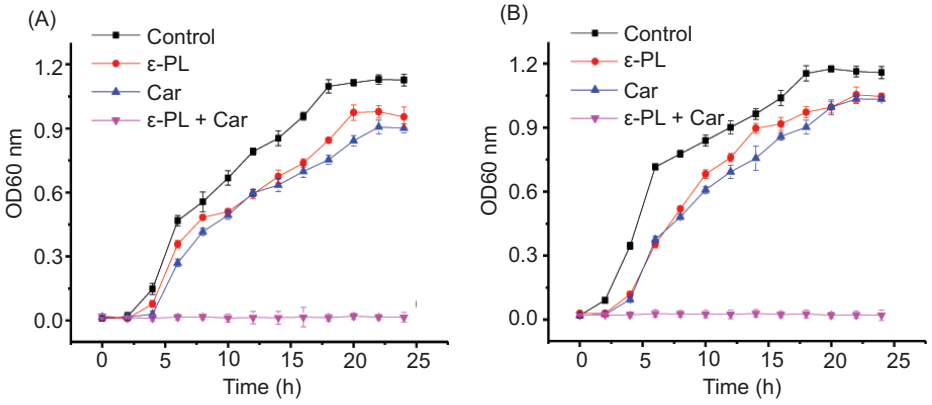

Figure 1. Growth dynamics curves for Car and ε-PL alone or in combination against *E. coli* CICC 10664 (A) and *S. aureus* CICC 21600 (B) in LB. The strains were treated with 80 μg/mL Car and 6.25 μg/mL ε-PL alone or in combination. The starting inoculum contained 10^6 CFU/mL. Values are the means of three independent experiments with SD indicated by vertical bars.

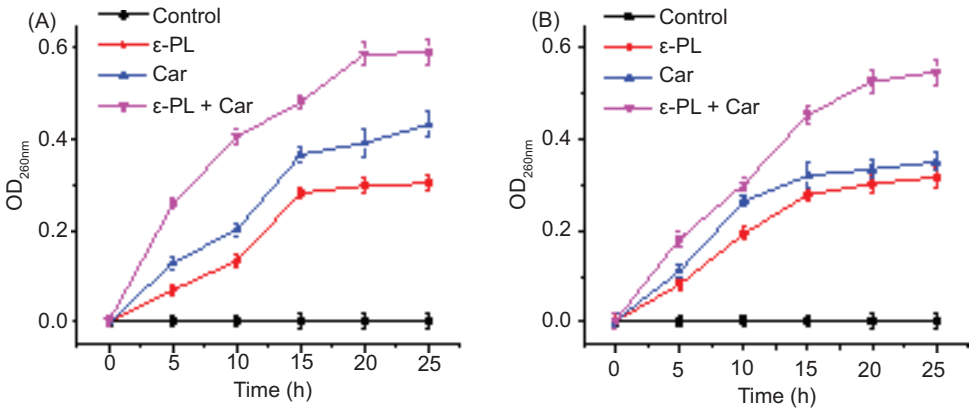

Figure 2. Effects of Car and ε-PL alone or in combination at 1 × MIC on the UV absorption at 260 nm of *E. coli* CICC 10664 (A) and *S. aureus* CICC 21600 (B) at different time points. Error bars are ± SD of the means.

DNA. It cannot pass through the cell membrane of living cells, but it can pass through damaged cell membranes and bind to nucleic acids in the nucleus. PI is embedded in double-stranded DNA and releases red fluorescence. The dot plots of *E. coli* cells stained with PI are shown in Figure 3.P2 zone is PI positive (PI+). Compared with the control group (Figure 3A1), the ε-PL (6.25 μg/mL) treatment group and the Car (80 μg/mL) treatment group yielded 2.1 and 29.8% *E. coli* deaths, respectively (Figure 3B1 and C1). However, ε-PL$^+$ Car treatment caused up to 62.4% *E. coli* deaths (Figure 3D1). Compared with the control group (Figure 3A2), ε-PL (6.25 μg/mL) and Car (80 μg/mL) treatment induced 5.2 and 12.2% *S. aureus* deaths, respectively (Figure 3B2 and C2). However, ε-PL$^+$ Car treatment caused up to 58.3% *S. aureus* deaths (Figure 3D2). The results showed that the combination of ε-PL + Car caused severe cell damage and that ε-PL could significantly improve the antibacterial effect of Car against *E. coli* and *S. aureus*.

Inhibition of respiratory chain dehydrogenase in pathogenic bacteria

The effect of bacteriostatic agents on cell membrane metabolic activity was studied by measuring respiratory chain dehydrogenase activity. The effect of ε-PL/Car on respiration chains dehydrogenase activity of *E. coli* and *S. aureus* cells are shown in Figure 4. The results showed that the respiratory-chain dehydrogenase activity in

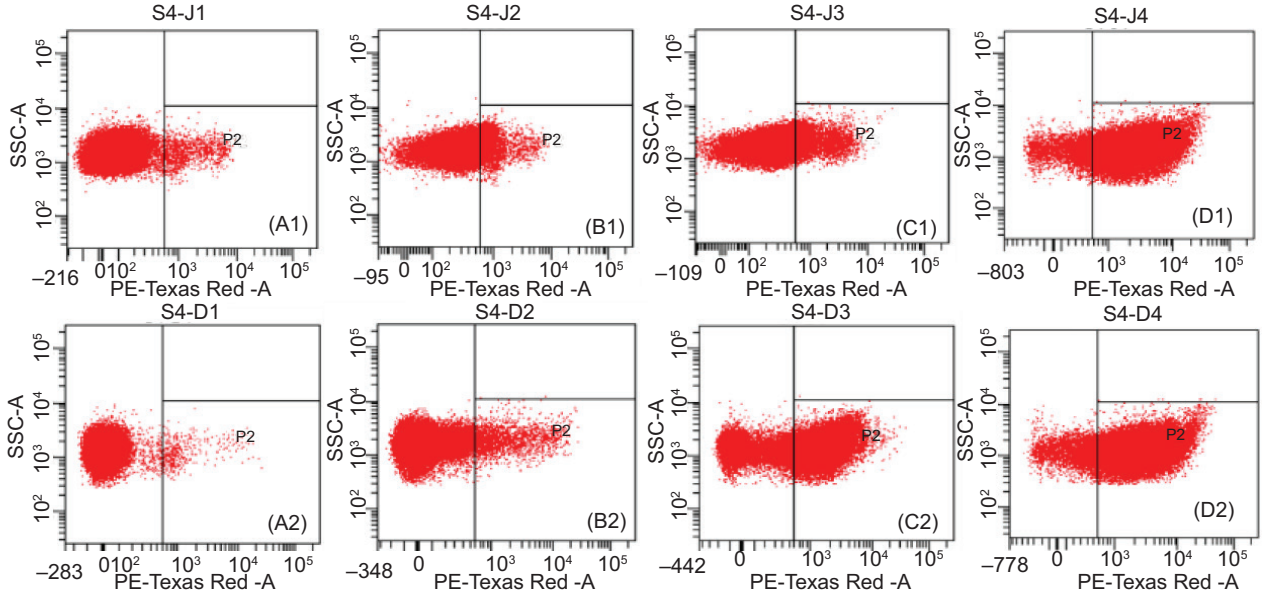

Figure 3. Flow cytometry dot plots of *E. coli* CICC 10664 cells (A1-D1) and *S. aureus* CICC 21600 cells (A2-D2). A1 and A2, untreated cells; B1 and B2, cells treated with ε-PL; C1 and C2, cells treated with Car; D1 and D2, cells treated with Car and ε-PL. Car concentration, 80 μg/mL; ε-PL concentration, 6.25 μg/mL.

the cells treatment for 6.25 μg/mL ε-PL was the same as that in without treated cells (control A). In addition, enzyme activity in cells treated with 80 μg/mL Car was slightly decreased, compared to the untreated group. Interestingly, 6.25 μg/mL ε-PL and 80 μg/mL Car inhibited 85% of respiratory chain dehydrogenase activity in *E. coli* and 61.6% of it in *S. aureus*. The result shows that the ε-PL/Car combination has a synergistic antibacterial effect on *S. aureus* and *E. coli*.

Thus, the reduction in the respiratory chain dehydrogenase activity caused by ε-PL/Car might inhibit normal respiration in cells, hinder bacteria growth, and even kill bacteria. The results suggested that ε-PL and Car exerted synergistic antibacterial effect against *E. coli* and *S. aureus*.

SEM observations

Pathogenic bacteria (*S. aureus* and *E. coli*) were treated for 3 h with ε-PL and Car alone or in combination using relevant MIC values (Car 80 μg/mL Car and 6.25 μg/mL ε-PL). Bacteria were then observed by SEM to investigate the resulting morphological changes. SEM was used to examine the cell surface. Nontreated cells were intact (regular rod) and exhibited a smooth surface, as noted in Figure 5A1. In contrast to the control, *E. coli* cells treated with ε-PL had irregular, withered, and coarse surfaces (Figure 5A2), indicating that cytoplasmic material might be released into the extracellular medium. Cells treated with Car exhibited slight surface damage (Figure 5A3), while Car + ε-pL treatment caused extensive surface damage to most cells. Specifically, both severe cell surface depression and cytoplasmic atrophy were observed (Figure 5A4). Untreated *S. aureus* exhibited intact cell membranes and plump round cells (Figure 5B1). The surface of some of the cells treated with ε-PL and cells treated with Car were damaged, wrinkled, and slightly irregular (Figure 5B2 and B3), while ε-PL + Car treatment caused extensive surface damage to most cells, including a sunken cell surface and shrinking cytoplasm (Figure 5B4). SEM observations confirmed that the structural integrity of the cells was damaged, and considerable morphological changes were noted in *E. coli* and *S. aureus*. The antibacterial effects of Car combined with ε-PL could be attributed to their pronounced deleterious effects on the bacterial cell membrane, potentially causing leakage of intracellular substances and complete cellular destruction.

Discussion

Effective food biopreservatives are needed to enhance food safety and quality in the food industry. Naturally produced antimicrobial compounds are acceptable agents to prevent the growth of undesirable organisms in food products. Carvacrol is a kind of monoterpenoid phenol derivative, primarily found in essential oils of herbs such as thyme and oregano. The biological properties of carvacrol have been extensively studied to prove its potential use in food preservation. However, the application of Car is limited due to the adverse sensory reaction caused by the amount of Car. The combination of Car with other antibacterial ingredients not only reduces the dosage and cost of Car but also widens its antibacterial spectrum and improves its application efficiency (Sharma *et al*., 2020; Wijesundara *et al*., 2021). This study verified the antibacterial effects of carvacrol and ε-PL alone, and in combination with *E. coli* and *S. aureus*. The synergistic effect of Car and ε-PL enhances their antibacterial activity

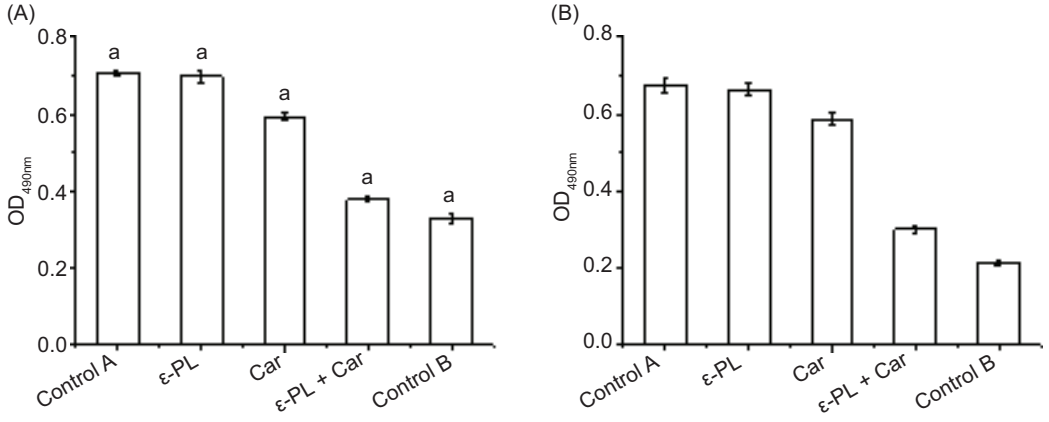

Figure 4. Inhibition of pathogenic bacteria respiratory chain dehydrogenase. (A) *E. coli* CICC 10664, (B) *S. aureus* CICC 21600. Control A: Live bacteria control. Control B: Dead bacteria control. The strains were treated with 80 μg/mL Car and 6.25 μg/mL ε-PL, alone or in combination. The starting inoculum was 10^6 CFU/mL. Values are the means of three independent experiments with SD indicated by vertical bars.

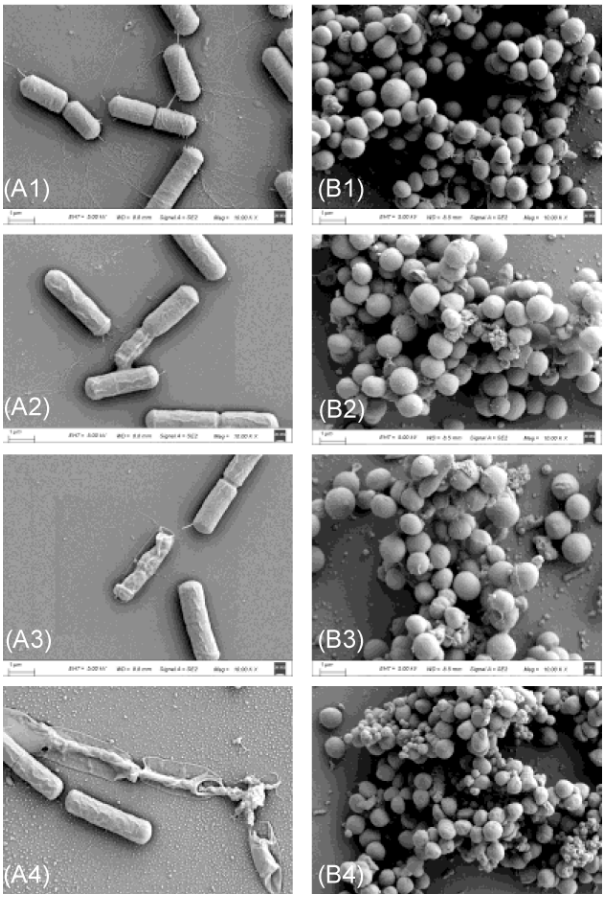

Figure 5. SEM observation of *E. coli* CICC 10664 cells (A1–A4) and *S. aureus* CICC 21600 cells (B1–B4) exposed to Car and *ε*-PL alone and in combination. A1 and B1, untreated cells; A2 and B2, cells treated with *ε*-PL; A3 and B3, cells treated with Car; A4 and B4, cells treated with Car and *ε*-PL.

($P < 0.05$). The MIC values of Car were greater than those for ε-PL against *E. coli* and *S. aureus*. The MIC values of ε-PL against *S. aureus* and *E. coli* were reported to be 31.25 - 62.50 μg/mL and 15.63 - 31.25 μg/mL respectively. MIC values of 75–375 μg/mL Car were reported against *S. aureus* and *E. coli* (Guarda *et al*., 2011). The combinations of citral and carvacrol, cinnamaldehyde and thymol, carvacrol and eugenol, thymol and eugenol have also been reported to have synergistic effects (Pei *et al*., 2009; Zanini *et al*., 2014). The library of natural substances with synergistic effects should be expanded in the future.

Several studies have reported the antibacterial effects of Car or ε-PL on pathogen growth when used in combination with other chemical compounds. Cao *et al*. (2021) researched the synergistic effects of carvacrol, citral, thymol, and caprylic acid, and the results indicated that the combination of citral and carvacrol showed a synergistic effect in inhibiting the growth of *Cronobacter sakazakii CICC 21544* (Cao *et al*., 2021). De Souza *et al*. (2020) analyzed the synergistic antimicrobial action of chitosan-gelatin-based active biopolymers combined with essential oils (EOs) against foodborne microorganisms, *S. aureus* and *E. coli strains*. Fang *et al*. (2015) evaluated the antibacterial effect of the combination of ε-PL and nisin against *Enterococcus faecalis* strains. The combination of ε-PL and nisin showed synergistic antibacterial activity against three *Enterococcus* strains. In the present study, our results indicated that Car and ε-PL are bacteriostatic agents with synergistic effects, which could be used for the control of pathogenic microorganisms. The activity of EOs and their components can affect both the cell envelope and cytoplasm. Their hydrophobic nature allows them to penetrate bacterial cell membranes and alter membrane structure and function (Filomena *et al*., 2013). Car is a hydrophobic compound that affects cell membranes by changing fatty acid composition, subsequently affecting membrane fluidity and permeability (Swamy *et al*., 2016). This action can lead to the disruption of proteins, DNA, RNA, or polysaccharides, depolarizing their potential and resulting in the death of the target microorganisms (Wu *et al*., 2009). Thus, the leakage of intracellular substances is an important sign of cell membrane damage. ε-PL exhibits a wide antimicrobial spectrum against Gram-negative and Gram-positive bacteria, yeasts, and molds. Different from the antibacterial mechanism of car, ε-PL mainly hinders DNA replication through insertion into the double helix structure, thereby inhibiting the growth of pathogenic bacteria (Liu *et al*., 2015). In the present study, the following ergistic antibacterial mechanism of Car and ε-PL has been proposed. Car rapidly destroys fluidity and permeability of bacteria membranes by changing fatty acid composition, and subsequently ε-PL enters the cell, interacts with DNA, and eventually hinders DNA replication. Synergizing the action of different bacteriostatic agents is one of the strategies to effectively control pathogenic bacteria; this reduces the use of each bacteriostatic agent in food. It is believed that Car might bind to cell membranes and then penetrate into the phospholipid bilayer. In this study, it is observed that the damage to the cell membrane permeability of the target strain caused by the synergistic effect of Car and ε-PL is their main action. Content of UV-absorbing materials released at 260 nm showed that both Car and ε-PL act on the lipopolysaccharide layer in *E. coli* and *S. aureus*, and change the permeability of the outer cell membrane. These results are consistent with those previously reported by Lv *et al*. (2011). In addition, combined with the result of reduced dehydrogenase activity in the cellular respiratory chain, it is speculated that Car might also alter membrane permeability by destroying the electron transport system, leading to an increase in the permeability of the inner membrane, which in turn leads to the leakage of intracellular substances. To clarify the morphological changes of cells

caused by Car and ε-PL, the alterations to bacterial membrane integrity were examined by SEM. Pathogenic bacteria (*S. aureus* and *E. coli*) exposed to ε-PL and Car alone or in combination using relevant MIC for 3 h showed degenerative changes of cytoplasmic membranes, and this leads to leakage of intracellular contents.

Conclusion

In the present study, we evaluated the antibacterial activity of the combined biocides (carvacrol and ε-polylysine) to test the potential of this new group of food preservatives in preventing food-borne pathogens. The results revealed that the combination of Car and ε-PL displayed good synergistic antibacterial activities against *E. coli* and *S. aureus* strains, increasing the effectiveness of either antibacterial agent alone. Furthermore, these results indicated that the application of a combination of ε-PL and Car can not only effectively control the number of *E. coli* and *S. aureus* but also reduce the amount of the two natural preservatives, ε-PL and Car. In addition, preliminary studies on the mechanism of action have shown that cell morphology and physiology are damaged by Car and ε-PL, resulting in the loss of intracellular components and the death of pathogens. Taken together, the combination of ε-PL and Car is an effective natural antibacterial agent, which has shown great application potential in the control of *E. coli* and *S. aureus* contamination.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

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