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Note

Thymol and eugenol in essential oils enhance phage endolysin LysECP26-mediated cell wall disruption of *Escherichia coli* O157:H7

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Abstract To enhance phage endolysin-mediated cell wall disruption of *Escherichia coli* O157:H7, the cells were co-treated with aromatic compounds, namely thymol or eugenol, found in essential oils and endolysin LysECP26. Interestingly, the minimal inhibitory concentrations of LysECP26 was four times lower when used in combination with either of the two compounds than when it was used alone. This synergistic activity was also confirmed by viable cell counting. Within 1 h of LysECP26 and eugenol or thymol co-treatment to the cells, there was a 2.3 or 3.8 log CFU/mL reductions, respectively. Additionally, field emission scanning electron microscopy showed cell wall disruption and severe morphological alterations of the cells in case of the combination treatments. Therefore, endolysin and thymol or eugenol co-treatment can help in developing efficient bio-control strategies against gram-negative pathogen *E. coli* O157:H7.

Keywords: bacteriophage, endolysin, thymol, eugenol, *Esherichia coli* O157:H7

Introduction

Many researchers have attempted to develop new antimicrobial agents based on the cell wall hydrolase properties of endolysins (Schmelcher et al., 2012). Such previous studies have focused on endolysins that target gram-positive bacteria, while gram-negative-targeting endolysins have not been much researched (Briers et al., 2015). This is because in gram-negative bacteria, penetration of endolysin to the peptidoglycan layer is prevented by the outer membrane (OM) (Fischetti, 2005). However, this barrier can overcome by treating the cell with OM permeabilizers (OMPs) or fusing membrane-permeable peptides to endolysin (Briers et al., 2015). Synergistic effects of OMPs and endolysins against gram-negative pathogens such as *E. coli*, *Salmonella*, and *Pseudomonas* have been shown previously (Oliveria et al., 2014; Oliveria et al., 2016). In another study, a molecular engineered endolysin, called Artilysin, was developed and successfully applied against gram-negative pathogens (Briers et al., 2014). With the addition of a combined peptide, the engineered endolysin was able to pass through OM and kill multidrug-resistant strains.

Antimicrobial compounds such as carvacrol, thymol, and eugenol in essential oils, which are extracted from plant materials, have been proposed as promising agents for a century (Perricone et al., 2015). The mechanisms underlying the antimicrobial activity of the compounds are diverse, but most are related to outer membrane disruption. The mechanisms of carvacrol are suggested as membrane structural and functional alteration, interference in synthesis and functionality of nucleic acids, cytoplasm coagulation and leakage of the vital constituents, metabolism imbalance, and the interruption of bacterial communication (Marinelli et al., 2018). Carvacrol has been applied to control *Staphylococcus aureus* with endolysin, however, thymol and eugenol known to disrupt OM of gram-negative bacteria were not applied with endolysin (Chang et al., 2017; Hyldgaard et al., 2012). Using these compounds with endolysin, a more effective strategy to inhibit the growth of gram-negative bacteria can be established.

Therefore, the aim of this study was to improve the antimicrobial activity of endolysin LysECP26 with the aromatic compounds for efficient biocontrol strategies against gram-negative pathogen *E. coli* O157:H7.

Materials and Methods

Recombinant protein preparation and minimal inhibitory concentration (MIC) determination

Recombinant LysECP26 was prepared as described previously (Park and Park, 2020). Briefly, *E. coli* C41(DE3) pRARE cells that contained pET23a-LysECP26, were cultured until an OD_{600} of 0.6 was achieved and induced with 1 mM IPTG at 37°C for 4 h. Then, LysECP26 was purified from the cells using Ni-NTA Superflow resin (Qiagen, Hilden, Germany).

To assess the synergistic effects of LysECP26 and the aromatic compounds of thymol and eugenol, a minimum inhibitory concentration (MIC) assay was conducted using the resazurin-based colorimetric assay, as previously described, with some modifications (Elshikh et al., 2016). Briefly, thymol (Duksan, Ansan, Korea) and eugenol (Sigma-Aldrich Co., St Louis, MO, USA) were dissolved in a 10% EtOH solution (pH 7.4) and serially diluted in 2× LB broth in a 96-well plate. Next, LysECP26 (1 μg/mL) or PBS buffer was added to each well. Then, each well

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was inoculated with exponentially growing *E. coli* 13930 (2 μL) and incubated at 37°C for 24 h. For MIC determination, 5 μL of resazurin (0.01% w/v) was added to all wells and incubated at 37°C for 1 h. After incubation, MIC was determined at the lowest concentration prior to the color change.

Evaluation of the antimicrobial activity of LysECP26 with thymol and eugenol

The synergistic antimicrobial activity of LysECP26 with thymol and eugenol against *E. coli* O157:H7 NCCP 13930 was assessed. Briefly, LysECP26 (1 μg/mL), 10% EtOH, thymol (8 μg/mL), and eugenol (32 μg/mL) were mixed with exponentially grown *E. coli* NCCP 13930 (~10^7 CFU/mL) and incubated at 25°C for 1 h. The bacteria were collected every 10 min and plated on the sorbitol-MacConkey agar (Oxoid, Basingstoke, Hampshire, UK) for counting viable bacterial cell (CFU/mL). The agar plates were incubated at 37°C and then individual colonies were counted.

Field emission scanning electron microscopy (FE-SEM) to assess morphological changes

E. coli O157:H7 cells were treated with the following antimicrobial agents for 1 h: LysECP26 (1 μg/mL)+10% EtOH, LysECP26 (1 μg/mL)+thymol (8 μg/mL), and LysECP26 (1 μg/mL)+eugenol (32 μg/mL), as mentioned above. After incubation, the cells were pre-fixed with 2.5% glutaraldehyde (Junsei Chemical, Tokyo, Japan) in PBS at 4°C for 24 h. Then, the pre-fixed samples were washed twice with distilled water and dried in a graded series of EtOH (50, 70, 80, 90, and 100%). They were then successively dehydrated with a graded series of hexamethyldisilazane (33, 50, 66, and 100%) (Sigma-Aldrich Co.). The dehydrated samples were examined using an FE-SEM (S-4700; Hitachi, Tokyo, Japan) at 10 kV and magnifications of 10,000×.

Statistical analysis

Triplicate experiments were done and the data were expressed as mean±standard deviation (SD). The results were analyzed by one-way analysis of variance (Dunnett's test, $p<0.05$) by GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

Synergistic effects of LysECP26 with thymol and eugenol against *E. coli* O157:H7

Previously, a combination of LysECP26 and organic acids was shown to inhibit the growth of *E. coli* O157:H7, however, these combinations did not show efficient bactericidal effects (Park and Park, 2020). To observe the synergistic bactericidal effect of endolysin and aromatic compounds, the combined treatment of the aromatic compounds and LysECP26 was conducted. Considering the optimum concentrations with LysECP26, the compounds were applied with and without LysECP26 (1 μg/mL) to *E. coli* O157:H7 NCCP 13930, and resazurin-based MIC assays were carried out (Fig. 1A). Both compounds showed bactericidal activities against *E. coli* O157:H7 NCCP 13930, but thymol (MIC 64 μg/mL) had a stronger effect than eugenol (MIC 256 μg/mL). The combination of LysCECP26 with the compounds showed higher bactericidal activities, suggesting that LysECP26 successfully reached the peptidoglycan after OM was disrupted by the compounds. The combination of LysECP26 and thymol was more effective than the combination of LysECP26 and eugenol, and MIC was four times

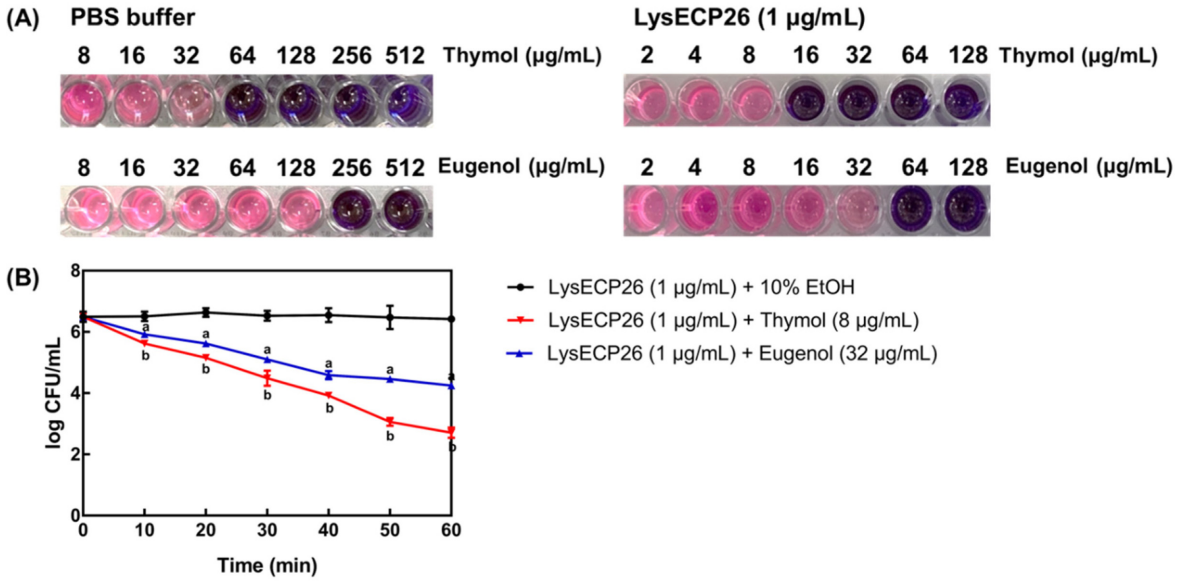

Fig. 1. Synergistic bactericidal activity of LysECP26 and essential oils against *E. coli* O157:H7 NCCP 13930. (A) Evaluation of the minimal inhibitory concentration (MIC) by a 96-well plate assay using resazurin. A change in color from pink to blue indicated the growth inhibition. *E. coli* O157:H7 cells were incubated with either thymol or eugenol along with PBS buffer or LysECP26 (1 μg/mL). (B) Time-kill curves of the LysECP26 and combinations of thymol and eugenol against *E. coli* O157:H7 NCCP 13930 cells. Black circle, LysECP26 with 10% EtOH (a negative control); red inverted triangle, LysECP26 with thymol; blue triangle, LysECP26 with eugenol. The mean values from triplicate measurements are plotted. Symbols indicate the significance of variances ($p<0.01$).

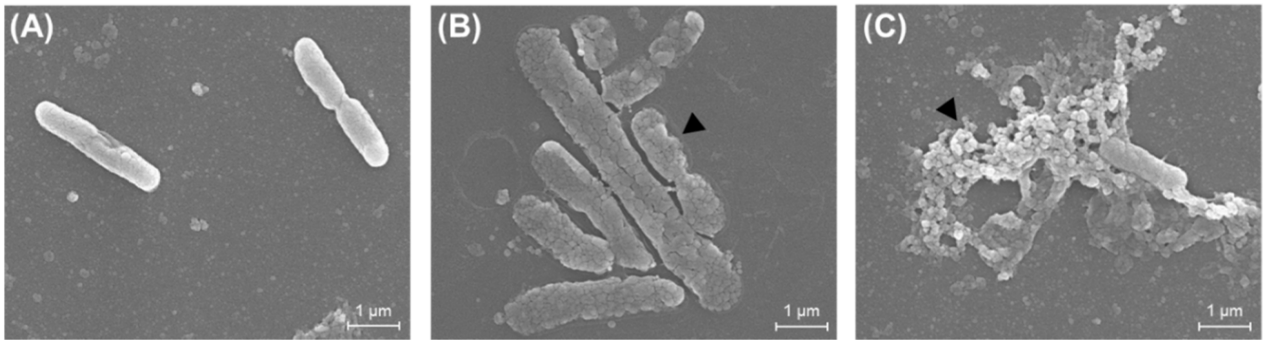

Fig. 2. Scanning electron microscopic analysis of the morphological changes caused by LysECP26 with thymol and eugenol on *E. coli* O157:H7 cells. FE-SEM images showing cells treated with (A) LysECP26 (1 μg/mL) and 10% EtOH; (B) LysECP26 (1 μg/mL) and thymol (8 μg/mL); (C) LysECP26 and eugenol (32 μg/mL). Magnification 10,000x and scale bar indicates 1 μm. Black arrowheads indicate cell debris likely due to cell lysis.

lower than that for the individual treatment. Such low concentration application of essential oils to foods might be another advantage to improve unique flavour coming from essential oils. To verify the biocontrol efficacy of the treatment, *E. coli* O157:H7 NCCP 13930 cells were treated with LysECP26 either alone or in combination with thymol or eugenol, and the viable cell numbers were monitored every 10 min (Fig. 1B). Treatment with LysECP26 and 10% EtOH had no antimicrobial activity, indicating that 10% EtOH, which was used as the solvent for essential oils, did not affect OM permeability. In contrast, treatment with LysECP26 and either compound efficiently lysed the *E. coli* O157:H7 cells and significantly reduced the number of viable cells in a time-dependent manner. LysECP26 with eugenol led to a 2.3 log CFU/mL reduction in the bacterial cells within 1 h. Treatment of LysECP26 with thymol showed faster, stronger antibacterial activity than LysECP26 with eugenol, and LysECP26 with thymol led to a 3.8 log CFU/mL reduction in the bacterial cells within 1 h. Considering that thymol and eugenol were applied by less than MICs, it might be concluded that thymol had higher OM disrupting activity than eugenol against *E. coli* O157:H7 NCCP 13930. It is previously shown that treatment with endolysin Cpl-7S and carvacrol reduces the viability of *E. coli* by 3 log units (Diez-Martinez et al., 2013). Another group reported that the combination of LysSA97 and carvacrol more efficiently reduces *S. aureus* counts than carvacrol only in a food environment by 2.0 log units within 3 h (Chang et al., 2017). Therefore, thymol was a better candidate OM disruptor that could be used with endolysins against *E. coli* than eugenol.

Morphology of *E. coli* O157:H7 treated by the combination of LysECP26 with thymol and eugenol

To observe the morphological changes caused by the antimicrobial action on cells by combination of LysECP26 and the compounds, *E. coli* O157:H7 NCCP 13930 cells were treated with LysECP26 and a compound as described above and then visualized ultrastructurally by field emission scanning electron microscopy (FE-SEM) (Fig. 2). As shown in Fig. 2A, the combination of LysECP26 and 10% EtOH did not cause any morphological changes in the *E. coli* cells. In contrast, treatment with LysECP26 and either thymol or eugenol caused cell wall disruption and serious morphological alterations (Fig. 2B and 2C). However, the patterns of cell destruction for the cells treated with the two compounds were different. The combination of LysECP26 and thymol destroyed the cells, leaving scratched and swollen structures, while the combination of LysECP26 and eugenol broke the cell into small pieces. High aromatic compounds such as carvacrol, eugenol, and thymol increase the membrane permeability leading to the loss of cell viability mainly by disruption of the cytoplasmic membrane to cause the passive flux (Trombetta et al., 2005; Yap et al., 2012). Thymol makes the perturbation of the lipid fraction of plasma membrane and leads to alterations of membrane permeability, which might make cell bigger by transporting water in the cell. Thymol is also known to inhibit ATP synthesis by impairing the citrate metabolic pathway to be antibacterial agent (Di Pasqua et al., 2006). Eugenol can alter the fatty acid profile in the cell membrane and lead to cell damage (Di Pasqua et al., 2006). LysECP26 appeared to have much easier access to the peptidoglycan layer with the aid of the compound-induced outer membrane disruption to destroy the peptidoglycan into smaller pieces. Taken together, these results suggest that supplementation with thymol or eugenol not only promotes the action of LysECP26 but also induces morphological changes in *E. coli* O157:H7 NCCP 13930 cells.

Therefore, a combination of LysECP26 and the aromatic compounds including thymol and eugenol showed better bactericidal performance than single-treatment approach. These phage lysis adjuncts would be needed to disrupt cell wall with endolysin and be helpful in developing efficient biocontrol agents against *E. coli* O157:H7.

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Conflict of interest

The authors declare that they have no competing interests.

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