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Lipoteichoic acids of lactobacilli inhibit *Enterococcus faecalis* biofilm formation and disrupt the preformed biofilm

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***Enterococcus faecalis*, a Gram-positive bacterium commonly isolated in patients with refractory apical periodontitis, invades dentin tubules easily and forms biofilms. Bacteria in biofilms, which contribute to recurrent and/or chronic inflammatory diseases, are more resistant to antimicrobial agents than planktonic cells and easily avoid phagocytosis. Although *Lactobacillus plantarum* lipoteichoic acid (Lp.LTA) is associated with biofilm formation, the effect of Lp.LTA on biofilm formation by *E. faecalis* is not clearly understood. In this study, we investigated whether Lp.LTA inhibits *E. faecalis* biofilm formation. The degree of biofilm formation was determined by using crystal violet assay and LIVE/DEAD bacteria staining. The quantification of bacterial growth was determined by measuring the optical density at 600 nm with a spectrophotometer. Formation of biofilms on human dentin slices was observed under a scanning electron microscope. *E. faecalis* biofilm formation was reduced by Lp.LTA treatment in a dose-dependent manner. Lp.LTA inhibited biofilm development of *E. faecalis* at the early stage without affecting bacterial growth. LTA from other *Lactobacillus* species such as *Lactobacillus acidophilus*, *Lactobacillus casei*, or *Lactobacillus rhamnosus* GG also inhibited *E. faecalis* biofilm formation. In particular, among LTAs from various lactobacilli, Lp.LTA showed the highest inhibitory effect on biofilms formed by *E. faecalis*. Interestingly, LTAs from lactobacilli could remove the biofilm preformed by *E. faecalis*. These inhibitory effects were also observed on the surface of human dentin slices. In conclusion, *Lactobacillus* species LTA inhibits biofilm formation caused by *E. faecalis* and it could be used as an anti-biofilm agent for prevention or treatment against *E. faecalis*-associated diseases.**

***Keywords*:** biofilm, *Enterococcus faecalis*, lipoteichoic acid, lactobacilli, apical periodontitis

Introduction

Enterococcus faecalis is a commensal Gram-positive bacterium that inhabits the oral cavity and gastrointestinal tract (Fisher and Phillips, 2009). *E. faecalis* can survive even under harsh conditions and has various virulence factors including lipoteichoic acid (LTA), gelatinase, surface adhesin, and aggregation substance protein (Kayaoglu and Orstavik, 2004; Fisher and Phillips, 2009). *E. faecalis* can act as an opportunistic pathogen causing urinary tract infection, bacteremia, and bacterial endocarditis (Madsen *et al.*, 2017). In particular, *E. faecalis* can easily invade dentinal tubules, colonize in microtubules, and form biofilms (Ramachandran Nair, 1987). Thus, *E. faecalis* is involved in the pathogenesis of refractory apical periodontitis once endodontic treatment has failed (Distel *et al.*, 2002).

Microorganisms produce an extracellular polymeric substance (EPS) matrix, which allows easy attachment to surfaces of various tissues, teeth, and medical devices and aggregation with other matrix particles (Kostakioti *et al.*, 2013). The surface-attached microorganisms are called bacterial biofilms (Kostakioti *et al.*, 2013). The life cycle of biofilms can be divided into three stages: attachment, maturation, and dispersion (Kaplan, 2010). Bacteria in biofilms are more resistant to antimicrobial agents than planktonic cells by inactivating or trapping antimicrobials (Stewart, 2015). Furthermore, biofilm bacteria can avoid phagocytosis and contribute to recurrent or chronic inflammatory diseases (Calo *et al.*, 2011). Accumulating reports have shown that *E. faecalis* biofilm formation inside dentinal tubules is a crucial factor in the etiology of refractory apical periodontitis (Duggan and Sedgley, 2007; Jhajharia *et al.*, 2015).

Previous studies have shown that the currently-available intracanal medicaments to eliminate bacteria have limited antimicrobial effectiveness and adverse effects including tissue damage and allergic diseases. Besides, resistance of enterococci to traditional antimicrobial agents causes nosocomial and chronic infections and contributes to pathogenicity (Arias *et al.*, 2010). Thus, combined therapeutics with antibiotics and biofilm blockers are needed for efficient treatment against chronic infections by enterococci. Lactobacilli were reported

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to have anti-biofilm activities against antibiotic-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Melo *et al.*, 2016; Shokri *et al.*, 2018). We previously reported that LTA of *Lactobacillus plantarum* inhibits *Streptococcus mutans* biofilm formation (Ahn *et al.*, 2018a). However, the effect of *L. plantarum* LTA (Lp.LTA) on *E. faecalis* biofilm formation has not been reported. Thus, we investigated whether Lp.LTA could inhibit *E. faecalis* biofilm formation.

Materials and Methods

Reagents and chemicals

E. faecalis ATCC 29212 and *Lactobacillus rhamnosus* GG ATCC 53103 were obtained from the American Type Culture Collection. *L. plantarum* KCTC 10887BP, *Lactobacillus casei* KCTC 3260, and *Lactobacillus acidophilus* KACC 12419 were obtained from the Korean Collection for Type Culture and Korean Agricultural Culture Collection, respectively. LTA was purified from *L. acidophilus*, *L. casei*, *L. plantarum*, and *L. rhamnosus* GG as previously described (Baik *et al.*, 2008). Brain heart infusion (BHI) agar and glucose were purchased from BD Biosciences. Crystal violet dye was purchased from Junsei Chemical Co., Ltd. The LIVE/DEAD BacLight Bacterial Viability Kit was purchased from Thermo Fisher Scientific. All chemicals were purchased from Sigma-Aldrich Inc. unless otherwise indicated.

Crystal violet assay

E. faecalis was grown overnight in BHI broth at 37°C. The culture was diluted 1:100 in BHI broth and incubated at 37°C for 6 h. The bacteria were incubated in 96-well cell culture plates (Nunc) at 37°C for 24 h. Biofilms were washed with 100 μl of phosphate-buffered saline (PBS) and stained with 1% crystal violet solution for 30 min. The wells were washed with PBS, and the crystal violet-stained biofilms were solubilized in 100 μl of dissociation buffer (95% ethanol and 0.1% acetic acid in water). The optical density at 600 nm was determined using a microplate reader (Molecular Devices).

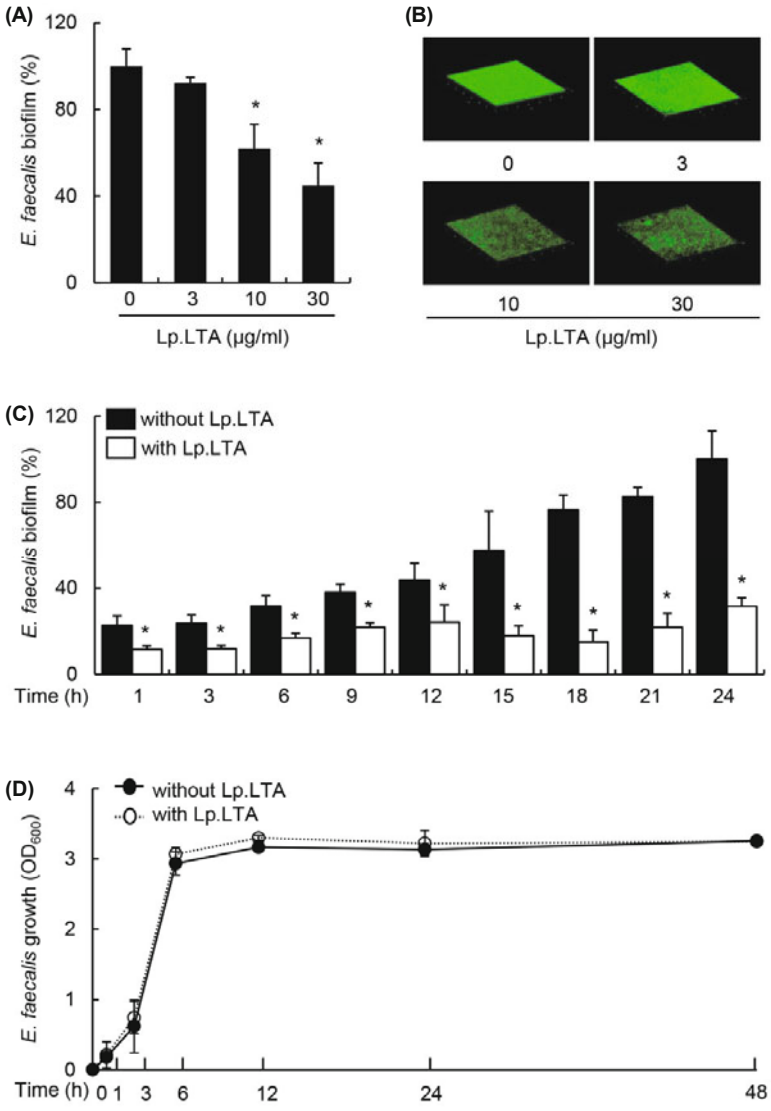

Fig. 1. *L. plantarum* lipoteichoic acid LTA (Lp.LTA) inhibits *E. faecalis* biofilm formation. (A) *E. faecalis* (1.4×10^9 CFU/ml) in 96-well cell culture plates was treated with 0, 3, 10, or 30 μg/ml of Lp.LTA for 24 h. *E. faecalis* biofilms were measured by using crystal violet assay. (B) *E. faecalis* (1.4×10^9 CFU/ml) was treated with 0, 3, 10, or 30 μg/ml of Lp.LTA. The bacteria were grown on a cover glass in 24-well plates at 37°C for 24 h. *E. faecalis* biofilms were stained with a LIVE/DEAD staining kit. *E. faecalis* biofilms were visualized under a confocal laser scanning microscope: Green (SYTO9; live cells), Red (propidium iodide; dead cells). (C) *E. faecalis* (1.4×10^9 CFU/ml) in the 96-well culture plates was treated with 30 μg/ml of Lp.LTA. The bacteria were grown at 37°C for various time periods. *E. faecalis* biofilms were determined by using crystal violet assay. (D) *E. faecalis* (1.4×10^9 CFU/ml) was treated with 30 μg/ml of Lp.LTA. The bacteria were grown at 37°C for various time periods. Growth curves were obtained by measuring the optical density at 600 nm (OD_{600}). $^*P < 0.05$. Results shown are representative of three similar experiments.

Confocal laser scanning microscopic analysis

E. faecalis was grown on slide glass (Muto Pure Chemicals Co. Ltd.) in a 24-well cell culture plate (Nunc) as previously described (Ahn *et al.*, 2018a). The biofilm was washed with PBS and stained with the LIVE/DEAD BacLight Bacterial Viability Kit containing SYTO9 and propidium iodide according to the manufacturer's instructions. Images were visualized under a confocal laser scanning microscope (LSM 800; Carl Zeiss MicroImaging GmbH).

Preparation of human dentin slices

Experiments with human dentin slices were approved by the Institutional Review Board of Seoul National University Dental Hospital, Seoul, Korea (CRI 17010). Human dentin slices were prepared as previously described (Ahn *et al.*, 2018a). Briefly, the surfaces of single-rooted premolars were cleaned using an ultrasonic scaler before producing cross-sections of 500 μm thickness with an Isomet precision saw (Isomet). The dentin slices were treated with 17% EDTA for 5 min and then treated with 2.5% sodium hypochlorite for 5 min. After neutralizing with 5% sodium thiosulfate, the dentin slices were autoclaved at 121°C for 15 min.

Scanning electron microscopic analysis

E. faecalis was grown on human dentin slices in the presence or absence of Lp.LTA and was visualized using a scanning electron microscope as previously described (Velusamy *et al.*, 2016; Ahn *et al.*, 2018a). Briefly, biofilms on human dentin slices were pre-fixed with a PBS solution containing 2.5% glutaraldehyde and 2% paraformaldehyde and washed with PBS. The biofilms were subsequently fixed with 1% osmium tetroxide for 90 min. After washing three times with distilled water, the biofilms were dehydrated by replacing the buffer with increasing concentrations of ethanol (70%, 80%, 90%, 95%, and 100% for 15 min each). The samples were dried with hexamethyl disilazane and coated with gold sputter. The image was visualized under a scanning electron microscope (S-4700, Hitachi). The images of *E. faecalis* biofilms were analyzed to determine the area of bacterial aggregates by using ImageJ software (National Institutes of Health).

Statistical analysis

All data are expressed as mean value ± standard deviation of triplicate samples. Statistical significance was analyzed using the *t*-test at $P < 0.05$.

Results

Lp.LTA inhibits *E. faecalis* biofilm formation

To examine the effect of Lp.LTA on *E. faecalis* biofilm formation, crystal violet staining and SYTO9/propidium iodide staining were performed. Crystal violet staining demonstrated that *E. faecalis* biofilm was decreased in a dose-dependent manner when incubated with Lp.LTA (Fig. 1A). SYTO9/propidium iodide staining also showed that Lp.LTA inhibited *E. faecalis* biofilm formation (Fig. 1B). These results indicate that Lp.LTA inhibits *E. faecalis* biofilm formation. Next, we examined how long the inhibitory effect of Lp.LTA on *E. faecalis* biofilm formation lasts. As shown in Fig. 1C, when *E. faecalis* was incubated with Lp.LTA, the inhibitory effect was first observed at 1 h and lasted up to 24 h. Notably, Lp.LTA did not affect *E. faecalis* growth (Fig. 1D), indicating that the inhibitory effect of Lp.LTA on *E. faecalis* biofilm formation was not due to the bacterial growth interference.

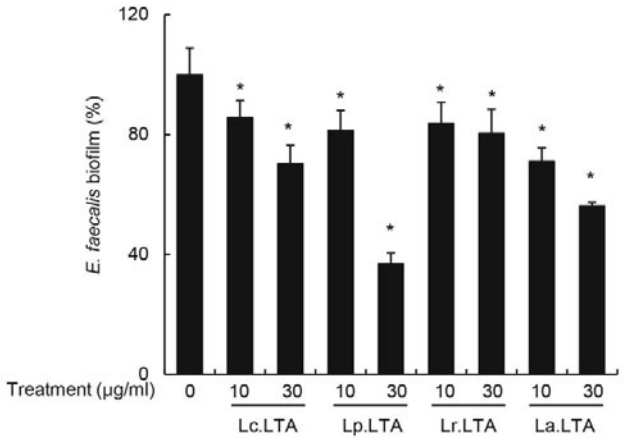

Fig. 2. LTAs from *Lactobacillus* species block *E. faecalis* biofilm formation. *E. faecalis* (1.4×10^9 CFU/ml) in 96-well cell culture plates were treated with 0, 10, or 30 μg/ml of LTA from *L. casei* (Lc.LTA), *L. plantarum* (Lp.LTA), *L. rhamnosus* GG (Lr.LTA), or *L. acidophilus* (La.LTA) for 24 h. The formation of *E. faecalis* biofilms was determined using crystal violet assay. *$P < 0.05$. One of three similar results is shown.

LTA from other *Lactobacillus* species also inhibits *E. faecalis* biofilm formation

Crystal violet assay was performed to determine whether *E. faecalis* biofilm formation was also inhibited in the presence of LTA from the other *Lactobacillus* species such as *L. casei*, *L. rhamnosus* GG, and *L. acidophilus*. As shown in

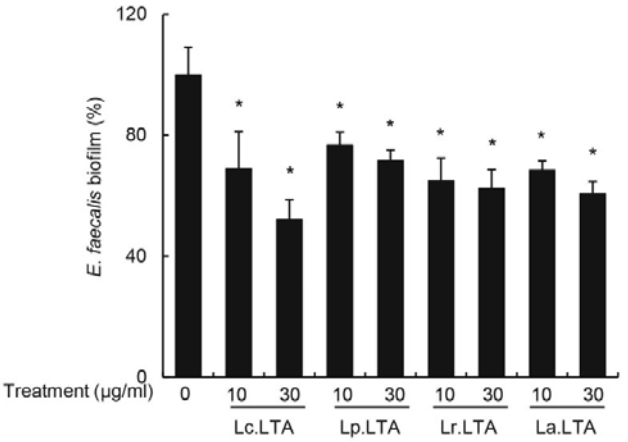

Fig. 3. Lp.LTA disrupts preformed biofilms. *E. faecalis* (1.4×10^9 CFU/ml) was grown in 96-well plates at 37°C for 24 h, followed by treatment with 0, 10, or 30 μg/ml of Lc.LTA, Lp.LTA, Lr.LTA, or La.LTA for 6 h. *E. faecalis* biofilms were determined by using crystal violet assay. *$P < 0.05$. One of three similar results was shown.

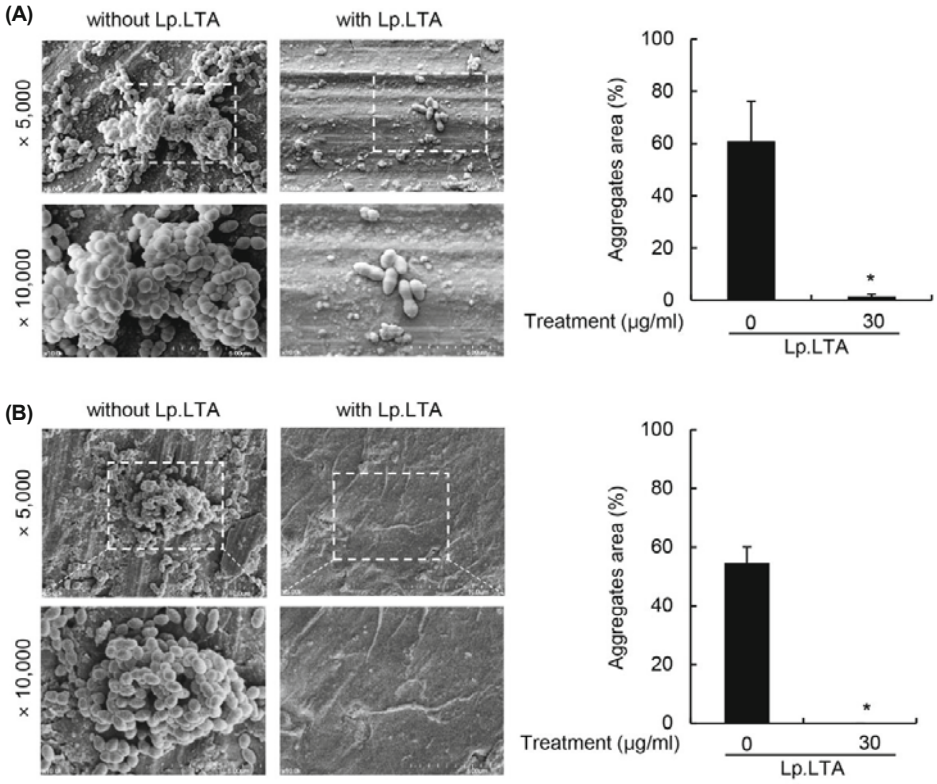

Fig. 4. Lp.LTA inhibits *E. faecalis* biofilm formation and disrupts *E. faecalis* biofilms preformed on human dentin slices. (A) *E. faecalis* (1.4×10^9 CFU/ml) on sterile human dentin slices was treated with 0 or 30 μg/ml of Lp.LTA. The bacteria were grown at 37°C for 24 h. (B) *E. faecalis* (1.4×10^9 CFU/ml) on sterile human dentin slices was grown at 37°C for 24 h, followed by treatment with 0 or 30 μg/ml of Lp.LTA for 6 h. The images of *E. faecalis* biofilms were captured under a scanning electron microscope. The area of bacterial aggregates was analyzed by ImageJ software. *$P < 0.05$. The result shown represents one of three independent experiments.

Fig. 2, *E. faecalis* biofilm formation was inhibited by all LTAs from the lactobacilli examined. The degree of inhibition by Lp.LTA at 30 μg/ml was superior to that of LTA from other *Lactobacillus* species. These results indicate that LTA from *Lactobacillus* species can inhibit *E. faecalis* biofilm formation.

LTA of *Lactobacillus* species disrupts biofilm preformed by *E. faecalis*

Next, we investigated whether lactobacilli LTA can affect the disruption of preformed biofilm. When treated with LTA from the lactobacilli including *L. casei*, *L. plantarum*, *L. rhamnosus* GG, and *L. acidophilus* for 6 h, the preformed *E. faecalis* biofilm was also reduced (Fig. 3). These results indicate that lactobacilli LTA not only inhibits *E. faecalis* biofilm formation, but also disrupts the preformed biofilm.

Lp.LTA inhibits *E. faecalis* biofilm formation on human dentin slices

We examined the inhibitory effects of Lp.LTA on biofilms formed on human dentin slices. *E. faecalis* biofilms in the presence or absence of Lp.LTA on human dentin slices were visualized using a scanning electron microscope. Lp.LTA significantly inhibited *E. faecalis* biofilm formation and disrupted the biofilm preformed on human dentin slices (Fig. 4A and B). These results indicate that LTA derived from *Lactobacillus* species could be a therapeutic candidate for blocking *E. faecalis* biofilm.

Discussion

Inhibition and/or elimination of *E. faecalis* biofilms in dentinal tubules is a potential therapeutic for refractory apical periodontitis. In particular, biocompatible anti-biofilm blockers are needed to control multi-drug-resistant pathogens such as *E. faecalis*. In this study, we demonstrated that Lp.LTA efficiently inhibits the formation of *E. faecalis* biofilms and even disrupted preformed biofilms. These results indicate that Lp.LTA could be an efficient treatment for refractory apical periodontitis and chronic infection caused by enterococci.

In the present study, we showed that LTA from various *Lactobacillus* species inhibits *E. faecalis* biofilm formation. Lp.LTA has the highest inhibitory effect on *E. faecalis* biofilm formation among various *Lactobacillus* species LTAs used in this study. Concordantly, *Lactobacillus fermentum* strains have been shown to inhibit biofilm formation caused by antibiotic-resistant strains of *P. aeruginosa* (Shokri *et al.*, 2018). We also previously reported that Lp.LTA inhibits the biofilm formation of *S. aureus*, a pathogenic bacterium causing serious infections including pneumonia, sepsis, and osteoarthritis (Ahn *et al.*, 2018b), and *S. mutans*, one of the major pathogens causing dental caries (Ahn *et al.*, 2018a). Thus, Lp.LTA might be a potential biofilm blocker inhibiting not only *E. faecalis* biofilm but also other bacterial biofilms.

In our study, Lp.LTA disrupted preformed *E. faecalis* biofilms. Similar to our results, lactobacilli and their culture supernatant have been shown to disrupt the preformed biofilms of pathogens such as *Vibrio* species and *Aggregatibacter actinomycetemcomitans* (Jaffar *et al.*, 2016; Kaur *et al.*, 2018).

The inhibitory effect of Lp.LTA was also observed in *S. aureus*-preformed biofilm (Ahn *et al.*, 2018b). Although further studies are needed to clarify the molecular mechanism for anti-biofilm activity of LTA, regulation of quorum sensing might be a potential mechanism since Lp.LTA increases the AI-2 level of *S. aureus* (Ahn *et al.*, 2018b), which is known to control the biofilm development by regulating the expression of biofilm-related genes or the production of molecules to break down EPS in *S. aureus* (Boles and Horswill, 2008; Yu *et al.*, 2012). In *E. faecalis*, Lp.LTA may possibly regulate the expression of enterococcal surface molecules such as sortase A and esp that are important for biofilm formation or produce enzymes to disrupt EPS by regulating quorum-sensing system (Chen *et al.*, 2016; He *et al.*, 2016).

Accumulating reports suggest that LTA, as a major cell wall component of Gram-positive bacteria, can induce innate and inflammatory responses (Kang *et al.*, 2016). LTA efficiently induces the expression of nitric oxide, chemokines, and cytokines (Baik *et al.*, 2008; Ryu *et al.*, 2009; Park *et al.*, 2013; Hong *et al.*, 2014). Likewise, previous studies have mainly focused on the immunostimulatory function of LTA. Recently, however, LTA has been investigated for its role as a regulator of immune response (Noh *et al.*, 2015; Kim *et al.*, 2017). Staphylococcal LTA attenuates lipopolysaccharide (LPS)-induced B cell proliferation (Kang *et al.*, 2018). Lp.LTA inhibits lipoprotein-induced IL-8 expression in human intestinal epithelial cells (Noh *et al.*, 2015) and the expression of TNF-α in LPS-induced endotoxin shock mice (Kim *et al.*, 2008). In our study, Lp.LTA efficiently inhibited the *E. faecalis* biofilm formation. Therefore, we suggest that bacteria-derived LTA could be an attractive therapeutic target for modulating immune responses and treating bacteria-mediated diseases.

In conclusion, the results from the present study demonstrated that Lp.LTA inhibits *E. faecalis* biofilm formation and disrupts the preformed biofilm. Furthermore, Lp.LTA efficiently inhibits biofilm formation at early stage and keeps the inhibitory effect ever afterward. These results provide evidence that LTA derived from *Lactobacillus* species could be a useful biofilm blocker to eliminate bacteria including *E. faecalis* and contribute to treatment of refractory apical periodontitis.

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Conflict of Interests

The authors declare no conflict of interests.

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