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Cover Page Footnote

The authors thank Faculty of Dentistry, Trisakti University, for invaluable support in this study. Authors also would like to thank Stella, SSi and Aradhea Monica Drestia, SSi from the Microbiology center of research and education (MiCORE) laboratory for their laboratory assistances.

ORIGINAL ARTICLE

Persistent Endodontics Pathogens Biofilm Inhibited by *Lactobacillus reuteri* Indonesian Strain

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ABSTRACT

Lactobacillus reuteri is known as probiotics that effective to improve oral health. **Objective:** To analyze the effect of *L. reuteri* Indonesian strain, towards *Enterococcus faecalis* and *Candida albicans* biofilm growth. **Methods:** This study was conducted using biofilm assay. Each *E. faecalis* ATCC-29212 and *C. albicans* ATCC-10231 were cultured in Brain Heart Infusion Broth, distributed into 96 well-microplate and incubated for 24h, 37°C, anaerobic condition for *E. faecalis* and aerobic condition for *C. albicans*. *L. reuteri* LC382415 (Indonesian strain) was cultured in de-Mann Rogosa Sharpe Broth and diluted into different concentrations (10⁸, 10⁶, and 10⁴CFU/mL) subsequently, each concentration distributed into biofilm well. Biofilm wells without probiotic was used as negative control. Biofilm mass were measured using crystal-violet dye at 490nm using microplate-spectrophotometer. Data was statistically analyzed by one-way ANOVA test, statistical significance set as $p < 0.05$. **Results:** Significant reduction of biofilm growth of *E. faecalis* and *C. albicans* after treatment with *L. reuteri*. The ideal concentration was found at *L. reuteri* 10⁸ CFU/mL with 79.2% *E. faecalis* biofilm reduction and *L. reuteri* 10⁴ CFU/mL with 62.5% *C. albicans* biofilm reduction compared to control ($p < 0.05$) set at 100%. The ANOVA test results showed that *L. reuteri* in all concentrations and all time periods in this study had the ability to inhibit biofilm growth of both species ($p < 0.05$) compared to negative control. **Conclusion:** *L. reuteri* Indonesian strain inhibit the biofilm growth of *E. faecalis* and *C. albicans*. This antibiofilm effect may be useful in preventing biofilm growth in root canal.

Key words: biofilm, *Candida albicans*, *Enterococcus faecalis*, *Lactobacillus reuteri*, probiotic

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INTRODUCTION

One of the common problems in the oral cavity is an infected root canal, with primary or secondary infection.¹ The infected root canal is a suitable medium for the growth of pathogenic microorganisms. This infection is caused by untreated dental caries, which allow the pathogenic microorganisms to spread to the apical region of the tooth and produce apical inflammation.² Without further treatment, this periapical infection will become an acute or chronic apical periodontitis, acute or chronic apical abscess, or cyst, as well as cellulitis. Causes can include oral cavity bacterial pathogens such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Fusobacterium nucleatum*, *Streptococcus spp.*, *Eubacterium*, and *Candida albicans*.^{2,3}

Enterococcus faecalis is an oral microbe most commonly found in infected root canals.^{4,5} *E. faecalis* has

a virulence factor, survival mechanism, and resistance that allow it to adapt to extreme environmental conditions, such acidic, alkaline, hypotonic, or hypertonic environments, over long periods. It is capable of surviving at low nutrient conditions and at temperatures ranging from 10 to 45°C.^{3,6}

This bacterium has an important role in biofilm development and biofilm formation contributes to bacterial resistance against antibiotics and phagocytosis.⁷ In a biofilm, the bacterial cells will develop a tolerance to antibiotics at 10–1000 times greater concentrations than are required to eliminate planktonic cells. For these reasons, *E. faecalis* has become a persistent organism. Although it makes up only a small proportion of the flora in untreated canals, it plays a major role in the etiology of persistent peri-radicular lesions after root canal treatment.⁷

In addition to bacteria, many *Candida* species, such as *C. albicans*, *C. dubliniensis*, *C. guilliermondii*, *C. krusei*, and *C. tropicalis*, are found in the oral cavity. However, the most commonly found species is *C. albicans*.⁸ This *Candida* is one of the normal flora found in the oral cavity in 25–75% of the population, and it also can be found in the human gastrointestinal tract.^{9,10} This species has the characteristics of an opportunistic pathogen: it is harmless in healthy individuals, but under certain conditions, it can cause infection in the oral cavity.^{10,11} The infection can be caused by a hormone imbalance, antibiotic overuse, long term use of immunosuppressants, and the ability of *C. albicans* to change into an invasive form.¹⁰ *C. albicans* has collagenolytic activity as one of its virulence factors, so it can use dentin as its nutrient source to increase its colonization of the root canal. *C. albicans* has a role in biofilm formation because of its ability to colonize the dentinal surface and to penetrate deep into the dentinal tubules.¹² This biofilm formation makes *Candida* sp. resistant to antifungal medications.¹³

The most common treatment for root canal infections such as apical periodontitis is endodontic treatment. Irrigation and medications are often used to reduce the load of pathogenic microorganisms in the root canal. The most widely used irrigation materials are sodium hypochlorite (NaOCl), chlorhexidine (2%), and EDTA (17%). These materials all inhibit the growth of *E. faecalis* and *C. albicans*, but each has its inadequacies. NaOCl is highly toxic if it is exposed to the periapical tissue, whereas chlorhexidine cannot dissolve most tissues and EDTA has no antibacterial activity.¹⁹ One of the most commonly used materials is calcium hydroxide (Ca(OH)₂), which has the potential to decrease the numbers of pathogenic microorganism in the root canal. However, *E. faecalis* and *C. albicans* may sometimes show resistance to this material, leading to the recurrence of infected root canals.^{5,14}

Probiotics are effective at enhancing oral health.¹⁵ One of the common commercial probiotics that has shown advantages in oral health is *Lactobacillus reuteri*,¹⁶ a Gram-positive bacterium that can produce the glycerol dehydratase enzyme to convert glycerol into reuterin in the presence of glycerols.^{17,18} Reuterin is a broad-spectrum antimicrobial agent that effectively inhibits both Gram-positive and Gram-negative bacteria, fungi, and protozoa by inhibiting DNA synthesis from pathogenic microorganisms.^{10,19} *L. reuteri* has been isolated from the saliva of Indonesian subjects and is registered in the Genbank with accession number LC 382415.²⁰

Previous studies indicated that *L. reuteri* DSM 17938 and *L. reuteri* PTA 5289 have antifungal activity against five of six *Candida* species commonly found in the oral cavity (i.e., *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*).¹⁰ *L. reuteri* also shows antibacterial activity against *E.*

coli ATCC 25922, *L. monocytogenes* ATCC 5313, *S. typhi* NCDC 113, and *E. faecalis* NCDC 115.²¹ Each *L. reuteri* strain has different ability on their effects as antibacterial agent as well as anti-inflammatory agent. Recent study showed the *L. reuteri* ATCC-55730 reduced the expression of the transcription level of interleukin (IL)-8 and human-beta-defensin (hBD)-2 as inflammatory factors.²² However, no studies have been conducted to determine the effectiveness of *L. reuteri* LC 382415 strain (Indonesian strain) against biofilms of *E. faecalis* and *C. albicans*. Thus, the objective of this study is to analyze the effect of *L. reuteri* LC 382415, towards *E. faecalis* and *C. albicans* biofilm growth *in vitro*.

METHODS

Bacterial culture

E. faecalis ATCC 29212 and *C. albicans* ATCC 10231 were obtained from MiCORE Laboratory, Faculty of Dentistry, Trisakti University and each of species was cultured in BHI broth medium for 24 h at 37°C under anaerobic conditions for *E. faecalis* and aerobic conditions for *C. albicans*. *L. reuteri* LC 382415 was cultured in Mann Rogosa Sharpe (MRS) broth medium for 24 h at 37°C, under anaerobic conditions.

Biofilm assay

E. faecalis ATCC 29212 and *C. albicans* ATCC 10231 were cultured, the optical density (OD) was measured with a microplate reader (490 nm) and diluted until reach bacterial concentration of Mc Farland 0.5 (1.5×10^8 CFU/ μ L). Each culture was homogenized with a vortex mixer and distributed to a 96-well plate and incubated for 48 h at 37°C under anaerobic conditions for *E. faecalis* and aerobic conditions for *C. albicans*. The culture supernatant was removed and the well-plate was washed with phosphate buffered saline (PBS). The *L. reuteri* LC 382415 culture was diluted (1×10^8 , 1×10^6 , and 1×10^4 CFU/mL), then distributed into a 96-well microplate. Positive and negative controls were also distributed into a 96-well microplate. NaOCl (5.25%) was used as a positive control for *E. faecalis* biofilm, and nystatine (100.000 IU) was used as a positive control for *C. albicans* biofilm. BHI broth was used as a negative control for *E. faecalis* and *C. albicans* biofilms. The decreasing biofilm numbers was observed after incubation in 37°C for 1, 3, 6, and 24 h under anaerobic conditions for *E. faecalis* and aerobic conditions for *C. albicans*. The biofilms were stained with crystal violet (0.5% w/v) by adding it to a 96-well microplate and incubating for 15 minutes. The dye solution was removed and 200 μ L of absolute ethanol was added to each well. The crystal violet dye remaining in the wells was counted as biofilm numbers. Biofilm inhibition was measured using crystal-violet dye at 490 nm and a microplate-spectrophotometer.²³ All treatment was done in triplicates and all bacterial medium culture were use as blank control.

Statistical analysis

The data were statistically analyzed by one-way ANOVA test; statistical significance was set as $p < 0.05$. Statistical calculations were performed with SPSS Statistics for Windows software version 20 (IBM, USA).

RESULTS

Study showed that there was a significant reduction of biofilm growth of *E. faecalis* after treatment with *L. reuteri*. The most effective concentration of *L. reuteri* was 1×10^8 CFU/mL (with OD value of 0.299) for a 79.2% reduction in *E. faecalis* biofilm after 1 hour when compared to the control (with OD value of 1.653) ($p < 0.05$) set at 100% (Figure 1). Result also showed that *L. reuteri* was effective on reducing the *C. albicans* biofilms growth significantly ($p < 0.05$). The most effective concentration for *L. reuteri* was 1×10^4 CFU/mL (with OD value of 0.880) for a *C. albicans* biofilm reduction of 62.5% in 1 hour when compared to the control (with OD value of 3.302) ($p < 0.05$) set at 100% (Figure 2).

DISCUSSION

E. faecalis and *C. albicans* are common microorganisms found in infected root canals, and they have the etiology of endodontic failure, also known as secondary root canal infection. Therefore, an antimicrobial factor is needed to inhibit the growth of these microorganisms. *L. reuteri* is a probiotic that has antimicrobial and antifungal properties and is useful for general human health as well as oral cavity health. Some of the advantages of *L. reuteri* probiotics are increased immunity, treatment of diarrhea in children, inhibition of *Streptococcus mutans* growth, which is one of the dental caries etiology, and prevention of gingivitis.^{24,25}

Previous research indicated that *L. reuteri* has a dehydratase enzyme that can transform glycerol into reuterin.^{17,18} Reuterin is a broad-spectrum antimicrobial factor that can inhibit Gram-positive and Gram-negative bacteria, fungi, and protozoa by inhibiting microbial DNA synthesis.¹⁷ The mechanisms by which probiotics inhibit microorganism biofilm formation is by increasing the epithelial barrier function and inducing mucus secretion, which simultaneously increases bacterial adhesion on the surface and inhibits pathogenic microorganism adhesion, through competition with pathogenic microorganisms to obtain nutrition, through interactions with receptors on the surface of host cells, through production of antimicrobial compounds, such as organic acids (including lactic acid and acetic acid) and bacteriocin, and by modulating the immune system of the host cells.²⁶

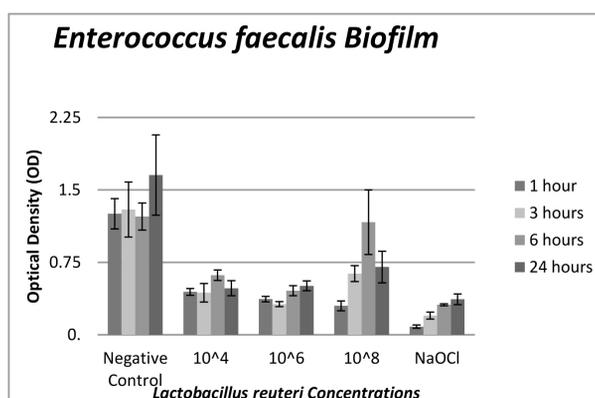


Figure 1. *E. faecalis* biofilm reduction after treatment with *Lactobacillus reuteri* in different concentration in 1h, 3h, 6h, and 24 hours incubation period (optical density). Biofilm without *L. reuteri* treatment was used as negative control and NaOCl as positive control.

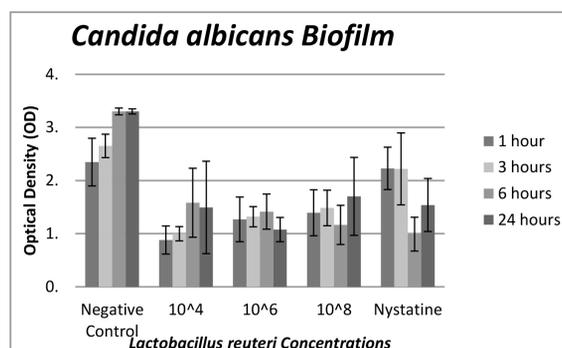


Figure 2. *C. albicans* biofilm reduction after treatment with *Lactobacillus reuteri* in different concentration in 1h, 3h, 6h, and 24 hours incubation period (optical density). Biofilm without *L. reuteri* treatment was used as negative control and Nystatine as positive control.

Lactic acid and acetic acid produced by *L. reuteri* can decrease the cellular pH in pathogenic microorganisms. The inhibition of pathogenic bacterial growth is caused by the ability acidic factors to penetrate the cell membrane, dissociate into the alkali surrounding the cell, and decreasing the pH in the cytoplasm.²⁷ The production of bacteriocins and hydrogen peroxide (H_2O_2) can damage the cell membrane of the pathogenic bacteria and inhibit DNA synthesis. Therefore, probiotics can prevent adhesion and further colonization of pathogenic microorganisms on the tooth surface.²⁸

Aside from bacteriocins and organic acids produced by most probiotic genera, some strains of *L. reuteri* also produce a uniquely active antimicrobial compound, reutericyclin, as their primary metabolite. Reutericyclin differs from most bacteriocins produced by lactic acid bacteria, because reutericyclin is actually a tetramic acid derivative that possesses both bacteriostatic and bactericidal activity against many Gram-positive and

Gram-negative bacteria, including *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile*.²⁹ A study conducted by Ganzle *et al.* (2000) reported that reutericyclin showed dose-dependent bactericidal activity, including cell lysis, against several bacteria. This study also reported that *E. faecalis* was the most sensitive indicator strain for reutericyclin, with a minimum inhibitory concentration (MIC) of 0.05 mg/L.³⁰ Although the precise mechanism of reuterin remains unclear, but the antimicrobial property was believed to be a result of damage to the microbial cytoplasmic membrane.³¹

The different incubation times in this study were intended to reveal the most effective time required by *L. reuteri* to decrease biofilm formation by *E. faecalis* and *C. albicans*. The effects on biofilm formation were related to the biofilm formation phases. In the first few minutes, microorganisms in the oral cavity attach to the dental surface or oral mucosa, and this stage is considered reversible. If the microorganisms are still attached to the surface, then the attachment will become stronger and irreversible in the next 2–4 h. Within 6–12 h, these microorganisms will form an extracellular polymeric substance (EPS) matrix that will protect the microorganisms from any medications. After 24 h, this biofilm will have matured. In this phase, the microorganisms in the biofilm will be more resistant to antimicrobial agents.³²

The use of probiotics for enhancing and maintaining oral health has been reported in many studies. Recent studies reported that consumption of probiotic significantly reduced the growth of *Streptococcus mutans* in saliva of orthodontic patients. This yogurt consumption could be used to reduce the prevalence of enamel demineralization among patients with fixed orthodontic appliances.^{33,34} Probiotics can also enhance oral health through immune system modulation. *L. reuteri* significantly reduced the production of the pro-inflammatory cytokine, interleukin (IL)-8, and induced the production of human beta defensin-2 in epithelial cells induced by infection with *S. mutans* and *P. gingivalis*. This immunosuppressive activity is considered beneficial to the host cell because it prevents prolonged inflammation and can speed the healing process of wounds related to oral bacterial infections.²²

CONCLUSION

Based on this study, it can be concluded *L. reuteri* Indonesian strain inhibit the biofilm growth of *E. faecalis* and *C. albicans*. This antibiofilm effect may be useful in preventing biofilm growth in root canal. However, further studies are needed to explore this result.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest related to the research

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