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Antibiofilm Activity of Parabiotic Reuterin on Acrylic Resin Plates

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Abstract*— Denture stomatitis is the most common debilitating problem among denture users worldwide. An alternative treatment could be natural probiotics. Probiotic *Lactobacillus reuteri* produces an antimicrobial substance called reuterin. The aim of this study is to evaluate effectiveness of parabiotic reuterin towards *Candida albicans* and *Staphylococcus aureus* biofilms on acrylic resin plates. The minimum inhibitory concentrations (MICs) of reuterin towards *C. albicans* and *S. aureus* were evaluated. Thereafter, standard preventive and therapeutic assays were performed on biofilms grown on acrylic denture plates. The effect of reuterin on biofilms was quantified with crystal violet staining, colony counting, and qPCR. The experimental data were analyzed by appropriate statistical tests. The MICs of reuterin towards *S. aureus* and *C. albicans* were 100µg/mL and 25µg/mL, respectively ($p<0.05$). Interestingly, reuterin exhibited antibiofilm activity against *S. aureus* and *C. albicans* at 100 µg/mL and eradicated the biofilms by 24 h incubation ($p<0.05$). Moreover, reuterin was effective against monospecies and mixed-species biofilms on acrylic plates. This pilot study showed that reuterin has an antibiofilm activity against *C.albicans* and *S.aureus* biofilms by reducing biofilms' number as much as 83% and 94.5 % respectively, in the denture acrylic plate model Hence, result will provide a basis for developing reuterin as a probiotic-based natural solution for denture stomatitis in the future.

Keywords—probiotic, reuterin, C. albicans, S. aureus, biofilms

I. Introduction

Dental and oral health problems are the most common health problems in the world. Particularly, Indonesia has a high percentage of dental and oral health problems. According to Indonesian Basic Health Research (RISKESDAS) in 2018, the most common dental health problems in Indonesia were toothache due to caries (45.3%), along with missing teeth due to extraction (19%), filled teeth due to caries (4.1%), and tooth loss (10.4%) [1]. Dental caries is the most common oral health problem worldwide [2].

Dental caries occurs as the cause of the complex biological interaction of acidogenic bacteria, fermented carbohydrates, and host factors, such as teeth and saliva. Bacteria in dental plaque, along with a decrease of salivary pH and fluoride, initiate dental caries [3]. Bacteria attached to the tooth surface will form a complex community known as a biofilm. It will also establish an extracellular matrix that surrounds the cells as they multiply in multicellular aggregates [4]. Once the carious lesion reaches the dentin, inflammatory changes would be visible around the pulp adjacent to the affected dentinal tubules. The carious lesion will develop into pulp along with subsequently increasing inflammation and ultimately causes tooth loss if not treated [5].

Tooth loss is a crucial measure of oral and dental health due to its long-term effects on health and social status [6]. Tooth loss impairs mastication, aesthetic functions [7], and will interfere with digestive systems and decreases the nutrients absorption for the body due to unchewed food.[8] Tooth loss is commonly found in geriatric patients, but it can also be found in children and young adults. Tooth loss in children and young adults affects the maxilla and mandible as well as molar relation [9].

Prevention of the destruction that occurs on dental hard tissue due to tooth loss requires prosthetic treatment, such as removable or fixed dentures [10]. Denture appliances are commonly used to treat patients with tooth loss. Removable dentures often caused oral health problems due to poor oral hygiene [11]. Poor denture hygiene causes *Candida albicans* (*C. albicans*) growth, which is an opportunistic pathogen in the oral cavity that causes denture stomatitis [12,13].

Candida albicans is an opportunistic pathogen because it can be the etiologic factor of diseases under certain conditions, such as immune deficiency in immunocompromised patients [14]. In general, *C. albicans* results in white lesions. It produces a highly structured biofilm that consists of several types of cells, including spherical yeast-forming cells and elongated cylindrical hyphae cells, in an extracellular matrix [15]. Some studies have provided information regarding co-isolation between *Staphylococcus aureus* (*S. aureus*) and *C. albicans* in several diseases related to biofilms, such as periodontitis, denture stomatitis, keratitis, and cystic fibrosis [16].

Staphylococcus strains on denture appliances cause increased activation of monocytes, thus making them more susceptible to the phagocytosis process. Based on previous research, phagocytosis process showed degradation of *S. aureus* biofilms in denture wearers [17]. The general denture stomatitis treatment is topical antifungal administration.

However, systemic administration can also be recommended for immunocompromised patients. Research has demonstrated the effectiveness of natural products for treating denture stomatitis [18].

Probiotics are defined within human nutrition as living microorganisms that are beneficial for humans, generally bacteria but sometimes yeast. Probiotics interact with intestinal microbes and host cells when taken in adequate amounts. These bacteria help balance the internal microbial and serve as a defense against pathogenic bacteria [19]. The consumption of probiotics decreases the number of pathogens in the oral cavity [20].

Lactobacillus reuteri (L. reuteri) produces antimicrobial compounds, such as organic acids, ethanol, and parabiotic called reuterin, which can inhibit the colonization of pathogenic microorganisms and change the composition of a commensal microorganism of the host. *L. reuteri* is also beneficial for the host immune system [21]. Parabiotic reuterin has an important role due to its active antimicrobial agent towards Gram-positive and Gram-negative bacteria, yeast, and protozoa as well as biofilms [22]. However, little knowledge is known regarding reuterin's effectiveness as an alternative denture cleaning agent. Therefore, this is the first study to establish the effectiveness of parabiotic reuterin isolated from Indonesian strain *L. reuteri* as an antibiofilm agent against *S. aureus* and *C. albicans* on acrylic resin plates.

II. Material and Methods

A. *Lactobacillus reuteri* culture and reuterin isolation

L. reuteri was cultured in De Man, Rogosa and Sharpe (MRS) (Oxoid, Hampshire) broth and incubated for 24 h in anaerobic environment at 43°C. Cell harvesting was performed with centrifugation at 5000 g for 15 min at 20°C. It was rinsed using phosphate-buffered saline (PBS) with pH 7.4 and then centrifuged at 5000 g for 15 min. The supernatant was discarded and resuspension of the cells was performed to reach concentration of 1.5 x 10^{10} colony forming units (CFU)/mL in MRS broth supplemented with 300 mM glycerol solution. Furthermore, it was incubated in anaerobic environment at 37°C for 3 h. After completed, centrifugation at 5000 g was performed for 15 min. The supernatant was filtered using a 0.22 µM size filter. The filtered supernatant was considered to be reuterin with the concentration of 100%.

B. Total protein concentration measurement

The Bradford test was performed to measure total protein concentration of the filtered supernatant [23]. Bradford assay was carried out by using the stock solution, working reagent, deionized H_2O with bovine serum albumin (BSA) (Merck, Darmstadt) (1mg/mL in deionized water), NaOH, protein sample of unknown concentration and sample buffer. Standard curve was established by making several dilutions of protein standard from 0 to 40 µg (0-40 µL of 1 mg/mL BSA) in final volume of 40 µL of deionized water. Eight concentrations of known protein (i.e., 0, 5, 10, 15, 25, 30, 35 and 40 µg) was measured on duplicate for the standard curve. 2 mL of Bradford working reagent (Merck, Darmstadt) was added to controls and samples, and were mixed thoroughly. Color was developed within 5 min at 37°C. The "blank" (0 µL of sample) was used as zero for spectrophotometer at 595 nm, and absorbance of controls and samples was recorded at 595 nm. Standard curve was determined by plotting the mean absorbance at 595 nm as function of standard protein concentration. Total protein volume was determined using standard curve above. Protein volume should be within linear range of standard curve. The protein concentration was measured for each volume, and concentrations were calculated for mean value. Total protein of samples can be read directly from standard curve or calculated using linear regression analysis to determine the slope of standard curve.

C. *Staphylococcus aureus* culture

S. aureus ATCC 25923 was cultured in anaerobic manner within Brain Heart Infusion (BHI) (Oxoid, Hampshire) broth for 24 h at 37°C. Bacterial cells were taken from centrifugation and suspended in PBS until it reached desired CFU/ml concentration of 1.5 × 10^8 CFU/mL prior to further research step.

D. *Candida albicans* culture

Candida albicans ATCC 10281 was cultured in sterilized Sabouraud Dextrose Broth (SDB) 5.6 pH (Oxoid, Hampshire) and incubated at 37°C for 24 h. Bacterial cells were taken from centrifugation and suspended in PBS until it reached desired CFU/ml concentration of 1.5 × 10^8 CFU/ml prior to further research step.

E. Minimum inhibitory concentration (MIC) test

The MIC test was performed in triplicate using the well-dilution method with 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.13 µg/mL, and 1.56 µg/mL concentrations. Phosphate buffered saline (PBS) was used as negative control. Chlorhexidine 0.2% (Minosep, Depok, Indonesia) and Nystatin (PT. Lapi Laboratories, Serang, Indonesia) were used as the positive control for *S. aureus* and *C. albicans*, respectively. Colonies were counted with total plate count method after incubation for 24 h at 37°C.

F. Biofilm assay

Biofilm assay test was performed in two ways: preventive and therapeutic. Preventive biofilm assay was performed using 96-well flat-bottom microplates (TPP, Trasadingen, Swiss). *S. aureus* and *C. albicans* cultures were inserted into each well of 96-well flat-bottom microplates along with 100 µL reuterin from *L. reuteri* isolate at concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL and incubated for 24 h at 37°C. Therapeutic biofilm assay was performed using 96-well flat-bottom microplates. *S. aureus* and *C. albicans* cultures were incubated for 24–48 h. After 1× wash with phosphate buffered saline (PBS) to clean unattached cells and leftover medium, the 96-well flat-bottom microplates were filled with 100 µL reuterin from *L. reuteri* isolate with 100 µg/mL, 50 µg/mL, 25 µg/mL, concentrations into each well and incubated for 8 h at 37°C. After incubation, biofilm staining was performed. The staining began by washing with PBS to release unattached cells and remove the bacterial growth medium. Next, 125 µL of 0.1% concentration crystal violet solution was added into each microtiter plate and incubated for 10–15 minutes at room temperature. The plates were washed using phosphate-buffered saline and then air-dried, followed by addition of 125 µL of ethanol absolute (Merck, Darmstadt). Spectrophotometer (SAFAS MP96, SAFAS, Monaco) with the wavelength of 595 nm was used quantify the biofilm absorbance value as total biofilm mass.

G. Test on acrylic resin plate

Acrylic resin plates were made at a size of 10 mm x 10 mm x 0.3 mm using heat curing process. First, the acrylic resin plate was soaked in water for 24 h to reduce residual monomer. The plate was then sterilized in an autoclave at 121°C for 15 min. Next, the plate was immersed into artificial saliva and washed using PBS. Then, the acrylic resin plates were inserted into growth medium containing *C. albicans* and/or *S. aureus* cultures and incubated for 24 h at 37°C to form biofilms on the acrylic plate surface. The acrylic resin plates were divided into five groups: group 1: 50 µg/mL concentration, group 2: 25 µg/mL concentration, group 3: 12.5 µg/mL concentration, group 4: Polident (GlaxoSmithKline, PT. Sterling Products, Jakarta, Indonesia) (positive control), and group 5: saline solution (negative control). The plates were soaked for 8 h for each group and washed using PBS. The cultured resin plates were vibrated in a vortex for 30 sec. The resin plates were removed and the supernatant for *C. albicans* was cultured into Sabouraud Dextrose Agar (SDA), *S. aureus* was cultured into BHI agar, and for mixed-species, it was cultured into BHI agar. The results were incubated for 48 h at 37°C and counted using colony counting methods.

H. DNA extraction

A volume of 1 mL bacterial culture in broth was centrifuged at 4500 g for 15 min. The supernatants were removed to obtain the bacterial pellet. Then, 1 mL phosphate-buffered saline (PBS) was added into the microcentrifuge tube with the pellet and homogenized using a vortex to wash the pellet from excess growth medium. The samples were centrifuged at 10,000 g for 10 min, and the supernatants were removed. Nuclease-free water (NFW) was added into the pellet, and the microcentrifuge tubes were sealed with Sherlock tube closures. The microcentrifuge tubes were incubated in thermo blocks for 20 min at 100°C. The microcentrifuge tubes were transferred into ice within 10 min, which is known as the heat shock process, to obtain bacterial DNA. The samples were re-homogenized with the vortex and centrifuged for 2 min at 10,000 g. The supernatants were transferred into new microcentrifuge tubes and stored at 4°C. If the tubes were evaluated for the presence of sedimentation re-centrifugation was done to obtain pure supernatants.

I. Real-time polymerase chain reaction (RT-PCR)

Colony counting confirmation was performed using RT-PCR platinum SYBR Green. The PCR solution was inserted into a 1.5 mL microcentrifuge tube. The solution consisted of 3 µL DNA template, 230 µL SYBR green (5 µL x 46), forward primers, and reverse primers (10 µM): 1 µL x 46. This study used specific primers for each microbe, which was a 16s rRNA gene. Primer sequence *S. aureus fwd* : 5'-CGA AAG CGT GGG GAT CAA AC-3' *rvs* : 5'-CCC AGG CGG TGT GCT TAA TG-3'. Primer sequence *C. albicans fwd* : 5'-CCC AGT CTT TCA CAA GCA GTA AAT-3' *rvs* : 5'-GTA AAT GAG TCA TCA ACA GAA GCC-3'.The forward and reverse primers were diluted with TE buffer to obtain a 10 µL concentration with a 9:1 ratio and homogenized using a vortex. The DNA of *L. reuteri* was diluted using nuclease free water (NFW) before the addition of the other solutions. The PCR solution was distributed into PCR tubes. Then, the diluted bacterial solution and 3 µL DNA sample were inserted into PCR tubes. The tubes were centrifuged at 1500 rpm speed for 1 min and transferred into RT-PCR for 120 min. The results were saved for analysis. The same steps were repeated to evaluate design plates. qPCR: Stage 1 (1 cycle): at 95°C for 10 min, Stage 2 (40 cycles): at 95°C for 15 sec, and at 56°C for 1 min, Melt curve: at 95°C for 15 sec, at 56° for 1 min, and at 95°C for 15 sec.

J. Data analysis

For the in vitro biofilm assay, we used the crystal violet staining method and colony counts. Colony counting method and real-time polymerase chain reaction (RT-PCR) as colony count confirmation were used for the acrylic plate examinations. The preceding data was analyzed using Kolmogorov-Smirnov normality test with a normal distributed value of $p>0.05$. Normally distributed data were analyzed using one-way ANOVA and post hoc (Tukey-HSD) with level significancy of $p<0.05$.

III. Results

A. Reuterin Isolation

The concentration of reuterin complex protein based on the results of the Bradford test obtained a mean of 200,993 µg/mL for reuterin of *L. reuteri* isolate LC 382415 at 100% concentration ("Table I").

TABLE I. Bradford Assay Results of 100% Concentrations of Reuterin from *Lactobacillus Reuteri* LC 382415

100% concentration of reuterin samples	Protein concentration (µg/mL)
LC 382415 - 1	188,865
LC 382415 - 2	199,436
LC 382415 – 3	214,667
Mean: 200,993	

B. Minimum inhibitory concentration of reuterin in planktonic bacteria

According to the MIC test, reuterin effectively inhibit the growth of *S. aureus* at 100 µg/mL concentration, and the growth of *C. albicans* at 25 µg/mL concentration. The minimum bactericidal concentration (MBC) of reuterin opposing *S. aureus* 200 µg/mL ("Fig. 1") whereas for *C. albicans* 50 µg/mL ("Fig 2").

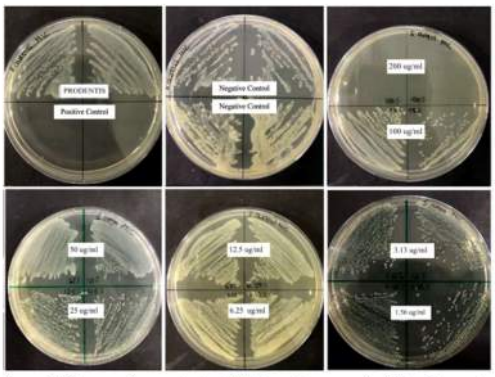

Fig. 1. Efficacy of reuterin in inhibiting the growth of biofilms using MIC test *S. Aureus*. Phosphate buffered saline (PBS) was used as negative control. Chlorhexidine 0.2% and nystatin were used as the positive control.

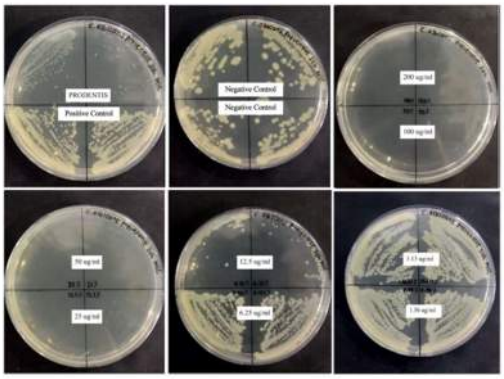

Fig. 2. Efficacy of reuterin in inhibiting the growth of biofilms using MIC test *C albicans*. Phosphate buffered saline (PBS) was used as negative control. Chlorhexidine 0.2% and nystatin were used as the positive control.

TABLE II. EFFICACY OF REUTERIN IN INHIBITING THE GROWTH OF *S. AUREUS* AND *C. ALBICANS*

	MIC	MBC
S. aureus	100 µg/mL	200 µg/mL
C. albicans	25 µg/mL	50 µg/mL

*MIC: *Minimum Inhibitory Concentration*
*MBC: *Minimum Bactericidal Concentration*

C. Biofilm Assay

Prevention assay. The result of biofilm prevention assay with crystal violet (CV) staining showed that reuterin at a concentration of 100 µg/mL works effectively towards *S. aureus and C. albicans* in preventing the growth of biofilms in vitro. Moreover, ensuing CFU counting, substantiate the preceding examinations ("Fig. 3").

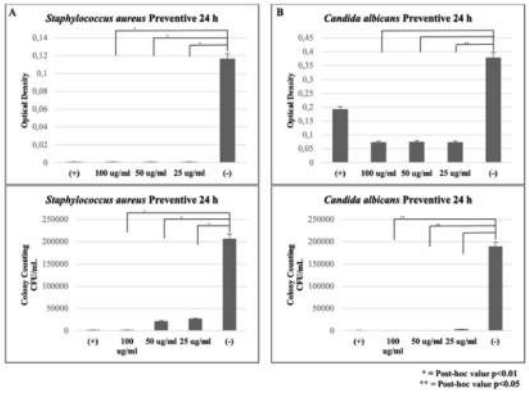

Fig. 3. Biofilm assay preventive result. Reuterin in inhibiting the growth and adhesion of *S. aureus* (A) and *C. albicans* (B) biofilms measured using crystal violet (optical density) and colony counting method. Phosphate buffered saline (PBS) was used as negative control. Chlorhexidine 0.2% and nystatin were used as the positive control. All treatment were done in triplicate.

Therapeutic assay. In biofilm therapeutic assay, the result of CV staining reveal that reuterin is notable in eradicating the biofilms of *S. aureus* and *C. albicans* at a concentration of 100 µg/mL. This study was performed in 8 h for the incubation time, thus the 100 µg/mL concentration of reuterin eradicate effectively in the period of incubation time. CFU counting result validate that 100 µg/mL concentration of reuterin works effectively in this study ("Fig. 4").

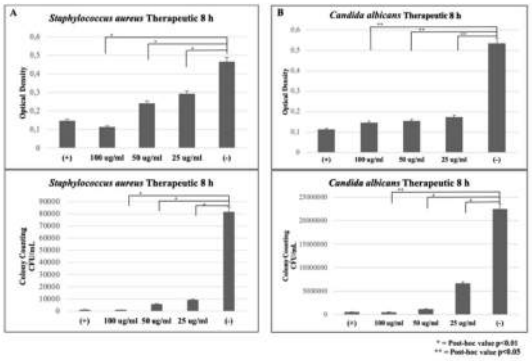

Fig. 4. Biofilm assay therapeutic. Reuterin in inhibiting the growth and adhesion of *S. aureus*(A) and *C. albicans* (B) biofilm measured using crystal violet (optical density) and colony counting method after 8 hours incubation. Phosphate buffered saline (PBS) was used as negative control. Chlorhexidine 0.2% and nystatin were used as the positive control. All treatment were done in triplicate.

D. Acrylic plate counting

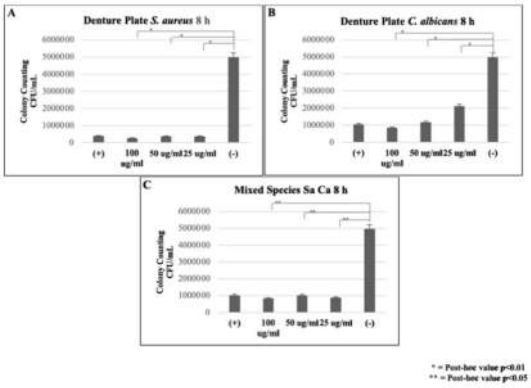

Fig. 5. The effectiveness of reuterin against *S. aureus* (A), *C. albicans* monospecies (B) and multispecies (C) biofilms on denture acrylic plate based on colony count measurements. Phosphate buffered saline (PBS) was used as negative control. Chlorhexidine 0.2% and nystatin were used as the positive control. All treatment were done in triplicate.

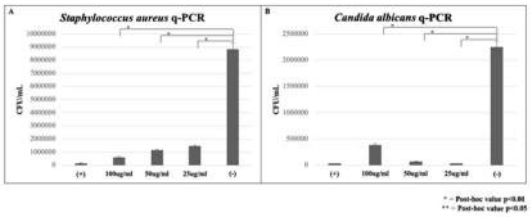

Fig. 6. The effectiveness of reuterin against *S. aureus* (A) and *C. albicans* (B) monospecies biofilms on denture acrylic plate based on qPCR result. Phosphate buffered saline (PBS) was used as negative control. Chlorhexidine 0.2% and nystatin were used as the positive control. All treatment were done in triplicate.

The results of mono species or mixed-species *S. aureus* and *C. albicans* test showed that reuterin inhibit mono species and mixed-species biofilms best at the concentration of 100 µg/Ml ("Fig. 5"). The real-time PCR result complementing the result ("Fig. 6").

IV. Discussion

Probiotics are microorganisms that can be beneficial to human health. The interest in probiotics usage to improve human health is increasing e.g. the content of dietary supplements. Probiotics improve human health by inhibiting the growth of pathogenic bacteria in the body [24]. *L. reuteri* is a probiotic bacterium that plays an important role in human health. It produces reuterin, which is an active agent against pathogenic bacteria [21].

The acrylic plate counting test proved that parabiotic reuterin is effective against mono species or mixed-species of *S. aureus* and *C. albicans*. This result showed a significant reduction of optical density (OD) values and colony counts. The MIC was at 100 µg/mL, 50 µg/mL, and 25 µg/mL concentrations. It can be presume that the higher concentration of reuterin has intended results to reduce mono species biofilm. The acrylic total plate count test results showed an optimal concentration of reuterin as a soaking solution against mono species and mixed species of *S. aureus* and *C. albicans* was 100 µg/mL. The biofilm assay and acrylic plate counting test results were compared with negative control, which was phosphate-buffered saline and culture medium without care. It can be inferred that reuterin has both effect in antibacterial and antifungal.

The parabiotic produce by probiotics *L. reuteri*, called reuterin with the chemical compound of 3-hydroxypropionaldehyde (3-HPA), plays an important role in probiotic *L. reuteri* mechanism of action [21]. The aldehyde group is very reactive to thiol and amine groups, causing the inactivation of proteins and micro molecules containing these groups [25]. This was proven by the MIC test results. The MIC tests showed, the value of *C. albicans* is higher than *S. aureus* with the concentration of 25 µg/mL due to the metabolism of glutathione (GSH) by *C. albicans*. GSH is a thiol compound that plays an important role in enhancing the resistance ability of *C. albicans* against antimicrobial and antifungal compounds [26]. Moreover, the pathogenic mechanism and virulence factor of *S. aureus,* which also includes a thiol group, namely bacillithiol (BSH), plays an important role in oxidative response [27]. BSH has a lower redox potential standard compared to GSH [26]. This result goes alongside the reuterin mechanism theory as an antimicrobial compound that reacts towards thiol groups. Another literature discovered that *L. reuteri* has the ability in regulating the inflammatory response such as IL-8 and HBD2 [28] and also proved that it is effective in inhibiting initial biofilm colonization [29].

After incubation and with the use of colony counts, the optimal reuterin concentration for *S. aureus* and *C. albicans* biofilm inhibition was obtained, which was at 100 µg/mL concentration. To eradicate mono species *S. aureus* and *C. albicans*, the optimal incubation time was 8 h, and the optimal concentrations were 50–100 µg/mL for *S. aureus* and 25–50 µg/mL for *C. albicans*. There are no specific data from previous studies to show that incubation time is a defining factor for the effectiveness of reuterin antimicrobial activity. However, based on the results of this study, it can be concluded that specific concentrations and 8 h of incubation time are the defining factors to eradicate biofilms.

The acrylic resin plate counting test result was compared to Polident (GlaxoSmithKline, Great Britain) as the positive control. Polident has been proven to be effective against *C. albicans*. Studies showed, that the use of cleaning tablets was proven to significantly reduce biofilms and their pathogenesis compared to water cleaning [11]. The use of reuterin on acrylic resin plates showed its effect against mono species and mixed-species colonization in between 25–100 µg/mL concentration of reuterin. Effects of reuterin in acrylic resin plates for both mono species and mixed-species was seen in 8 h of incubation time, assuming for the overnight usage. Co-isolation between these microorganisms is stronger and has greater virulence effects [30]. However, reuterin treatment showed the same results as Polident in reducing mono species and mixed-species biofilms. Quantification was performed using PCR to confirm detected bacterial DNA. This study showed that reuterin from *L. reuteri* isolate has an effect against *S. aureus* and *C. albicans* biofilms, either mono species or mixed-species.

V. Conclusion

In conclusion, the present study demonstrated that parabiotic reuterin from *L. reuteri* Indonesian strain is effective in inhibiting and eradicating monospecies and mixed species of *S. aureus* and *C. albicans* biofilms attachments on acrylic plate surfaces as much as 83% and 94.5 % respectively. Parabiotic reuterin isolated from Indonesian strain *L. reuteri* has a promising potential as a denture cleanser agent.

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