

Effect of Reuterin on Dual-species Biofilm *in vitro* of *Streptococcus mutans* and *Veillonella parvula*

Armelia Sari Widyarman^{1*}, Citra Fragrantia Theodora²

1. Department of Microbiology, Division of Oral Biology, Faculty of Dentistry, Trisakti University, Jakarta, Indonesia.

2. Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia.

Abstract

Probiotics have potential to restore homeostasis that may not be achieved with conventional treatment, by more effectively addressing the host-agent interaction. This study has been conducted to analyze the inhibitory effects of reuterin isolate *Lactobacillus reuteri* on the dual-species biofilm formation of *S. mutans* and *V. parvula in-vitro*.

V. parvula ATCC 10790T and *S. mutans* ATCC 25175T were used in biofilm formation. Reuterin isolated from *L. reuteri* LC382415 (1.5×10^{10} CFU/ml) were suspended in 5 ml of MRS broth containing glycerol (300mM) and incubated for 3h, at 37°C, anaerobically and confirmed by using SDS-PAGE and dot blot methods. 96-well flat-bottom microtiter plates biofilm assay were used to analyze the reuterin effect on dual-species biofilm. Bacterial suspensions were treated with different reuterin concentrations and biofilm inhibitory effect was observed periodically after 15 min, 3 h, 6 h, and 24 h incubation time. Data statistically analyzed by One-way ANOVA and level of significance set at $p < 0.05$.

52 kDa band visualized on SDS-PAGE agar confirmed the presence of reuterin. The reuterin inhibitory effect on dual-species biofilm of *S. mutans* and *V. parvula* were found in this study. Reuterin had the strongest activity on dual-species biofilm with the 100% concentration for 24h period. Meanwhile, the percentage reduction of biofilm after treatment with 50% concentration of reuterin for 6h could be reach until 91% reduction ($p < 0.05$).

This study is the first report to demonstrate that reuterin isolated from *L. reuteri* might be implicated to prevent the biofilm formation at early stages. Further studies are needed to explore this result with other oral pathogen.

Experimental article (J Int Dent Med Res 2019; 12(1): 77-83)

Keywords: Reuterin, Oral biofilms, *streptococcus mutans*, *Veillonella parvula*.

Received date: 27 July 2018

Accept date: 24 February 2019

Introduction

Biofilm is attached to a substratum or surface and it consists of many bacteria.¹ Infection cases become highly difficult to treat when bacteria succeed in forming a biofilm within a human host.² It is involved in the pathogenesis of oral diseases, such as caries, gingivitis, and periodontitis, through the development of multispecies of biofilm in the human dental plaque.^{3,4} Over 500 bacterial species have been isolated in human dental plaque.³ The interaction

of bacteria with each other was reported, such as communication by releasing specific molecules or using the metabolic end-products from other species.⁵ Biofilm is formed by the selective, reproducible, and sequential surface colonization of these species.^{6,7} Studying these species interactions would aid in elaborating on the mechanism of biofilm formation.⁵

Streptococcus sp. is a gram-positive coccus that initially⁸ adheres to the proteins and glycoprotein saliva adsorbed on the surfaces of the teeth.^{6,7} *Streptococcus mutans*, known as the main etiology of caries, has been reported for decades due to its acidogenity among *Streptococci* sp.^{9,10} Furthermore, *S. mutans* is the principal cariogenic pathogen present in dental plaque, co-existing with other species of bacteria^{3,11,12} in an interactive community of biofilm.¹³ *Veillonella* sp. are gram-negative cocci known to have a specific role as early colonizers

*Corresponding author:

Armelia Sari Widyarman, PhD

Department of Microbiology,

Division of Oral Biology, Faculty of Dentistry,

Trisakti University, Jakarta, Indonesia.

E-mail: armeliasari@trisakti.ac.id

in biofilm formation with *Streptococcus* sp.¹⁴ Oral *Veillonella* sp.,¹⁵ especially *Veillonella parvula*, are associated with severe early childhood caries,¹⁶ poor oral hygiene,¹⁷ intraradicular infections,¹⁸ abscesses,¹⁹ apical root canals,²⁰ and dental tubules.²¹

The definite interaction between *S. mutans* and *V. parvula* in biofilm formation is not fully explained. However, several factors may be involved in this interaction. *Veillonella* sp. cannot catabolize sugars; instead, they depend on lactic acid fermentation produced by *Streptococcus* sp. to form acetic and propionic acids, carbon dioxide, and hydrogen.^{22,23} In addition, the intrageneric coaggregation among *Streptococcus* sp., as well as the intergeneric coaggregation between *Streptococcus* sp. and *Veillonella* sp. might become highly important in the metabolic interaction of primary colonization on tooth surfaces.²⁴

With the increasing resistance to antibiotic drugs and an inclination among society to use “natural” therapies, the development of novel approaches for treating oral diseases that do not require conventional antimicrobial agents is necessary.²⁵ The restoration of microbial ecology balance has been proposed as one preventive approach, rather than the elimination of the disease-associated species.²⁶ This includes the use of probiotics to promote health-associated species or to support probiotic use with associated benefits.

Probiotics have been suggested as an alternative therapeutic option to control oral diseases. According to the Food and Agriculture Organization/World Health Organization (FAO/WHO), the term “probiotics” refers to “live microorganisms when administered in adequate amounts to deliberate a health benefit on the host.”²⁷ *Lactobacillus* sp. and *Bifidobacterium* sp. were mainly chosen for therapeutic treatment from among other probiotic bacteria.²⁸ *Lactobacillus reuteri* is an oral flora known as a probiotic, as it produces an antibacterial compound called reuterin. The structure of this broad-spectrum antimicrobial agent is related to 3-hydroxypropionaldehyde (3-HPA), and it is formed during fermentation with glycerol.^{29,30} Reuterin as an antibacterial has been stated to be effective against oral pathogenic bacteria,³¹ when consumed either through chewing gum or through *L. reuteri* ATCC 55730^T-containing yoghurts, and it results in the significant growth

inhibition of *S. mutans*.^{32,33} It also results in a reduction in *Porphyromonas gingivalis* and *Prevotella intermedia* in the subgingival pockets.³⁴

Many studies have demonstrated the significant inhibitory effects of *L. reuteri* on the *S. mutans* biofilm formation associated with dental caries.³⁵ However, there are no data on the inhibitory effects of reuterin on dual species in the early stages of biofilm formation. Hence, the objective of this study was to investigate the inhibitory effects of reuterin on the dual-species biofilm formation of *S. mutans* and *V. parvula* *in vitro*.

Materials and methods

Bacterial strains and culture condition

V. parvula ATCC 10790^T was cultured in brain heart infusion (BHI) blood agar (Thermo Fisher Scientific, Waltham, MA, USA) with 5% defibrinated sheep blood and 2% sodium lactate added, and it was incubated anaerobically (N₂: 80%, CO₂: 10%, and H₂: 10%) for 5 d. *S. mutans* ATCC 25175^T was cultured in tryptic soy broth (TSB) and tryptone yeast extract (TY) agar anaerobically for 3 d. The *Lactobacillus reuteri* LC382415 was cultured in the de Man, Rogosa, and Sharpe (MRS) broth medium and incubated anaerobically for 24 h.

Reuterin production

Reuterin was obtained through glycerol conversion using glycerol dehydratase produced by *L. reuteri*. *L. reuteri* LC382415 (Indonesian strain)³⁶ was collected by centrifugation at 4,000 × g for 10 min and washed with phosphate-buffered saline (PBS, pH 7.4) twice. Reuterin was extracted by following the method from Chen et al.³⁷ with modifications. Five ml of 300 mM glycerol in the MRS broth suspension of *L. reuteri* isolates from saliva (1.5 × 10¹⁰ CFU/ml) were incubated for 3 h anaerobically. After fermentation, the cells were collected by centrifugation at 4,000 × g for 10 min. The supernatant fraction was filtered by using a 0.22-µm pore size filter membrane (Millipore) and stored at 4 °C.

SDS-PAGE

The reuterin isolated from *L. reuteri* was separated by the sodium dodecyl sulfate (SDS)-poly-acrylamide gel electrophoresis (PAGE) method. The SDS-PAGE method was performed by following the method of Costas³⁸ with some modifications, using a 12.5% polyacrylamide gel in a MiniProtean SDS-PAGE apparatus (Bio-Rad, Hercules, CA, USA). After the SDS-PAGE method was performed, the gel was taken out and was stained using 0.05% Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, USA) before gel visualization.

Induction of specific antibody against reuterin in mouse model

The concentration of reuterin was determined using the Bradford protein assay, which resulted in a concentration of 202.454 µg/mL. In this study, (10) Swiss Webster (SW) mice were used as subjects to produce the anti-reuterin antibody, with 2,000 µg/mL of bovine serum albumin (BSA) used as a carrier to induce antibody production in the blood. The intraperitoneal injection method was carried out to administer reuterin into the subjects. In total, 200 µL of protein consisted of 185 µL (39.5 µg) of reuterin, 15 µL (30 µg) of BSA, and 100 µL of a booster composed of 96 µL (19.76 µg) of reuterin and 5 µL (10 µg) of BSA. The treatment was carried out for eight weeks, with the injection was repeated every two weeks. The blood of the mice was taken in the fourth, sixth, and eighth weeks of treatment from the orbital sinus of the mouse. Anesthesia was performed using ether, and 500 µL of blood was collected by thrusting a capillary tube in the middle corner of the mouse's eye. The blood was stored in a 1.5-mL microcentrifuge tube. To obtain the serum, the blood-containing tube was centrifuged for 5 min and then stored at 20 °C for the next step.

The Dot-blot assay

The dot-blot assay was carried out to confirm specific antibodies from SW mice treated with reuterin isolated from *L. reuteri* ATCC 55730 as an antigen following the method described by Renner et al. with modifications.³⁹ All steps were performed at room temperature. By using the nitrocellulose membrane, which was marked with

a pencil, a 5-µL sample was placed in the middle of the blotting area of the membrane, and the membrane was left to dry for a few minutes. Subsequently, the membrane was blocked with 5% blot containing TBS in a petri dish and agitated for 1 h. The membrane was rinsed with TBS three times, each for 5 min. Then, the membrane was blocked using a specific antibody against reuterin obtained from the mouse model that was diluted (1:250), agitated for 1 h, and rinsed with TBS for 5 min three times. The next step was the addition of 10 µL of a secondary antibody (anti-mouse IgG) into the membrane; then agitated for 1 h and rinsed with TBS. After rinsing, a developer reagent was added to the membrane, and the membrane was incubated in an orbital shaker for 30 min under a low light condition. After incubation, the membrane was rinsed three times for 5 min using sterile aqua dest.

SDS-PAGE to confirm specific antibodies from mouse model

This step was carried out once again to detect the presence of a specific antibody produced by the mouse model against reuterin by following the method of Costas with modifications.³⁸ The 4% stacking gel consisted of 0.5 M Tris-HCl (pH 6.8), 10% SDS, 30% polyacrylamide, H₂O, 10% APS, and TEMED. Meanwhile, the 12% resolving material consisted of 1.5 M Tris-HCl (pH 8.8), 10% SDS, 30% polyacrylamide, H₂O, and 10% APS. Next, resolving agar was inserted into the agar cast. After polymerization (hardening) for 30 min, stacking gel was added. Then, 30 µL of reuterin was added, along with 10 µL of native buffer, and it was heated for 5 min at 100 °C. After the polymerization of the stacking gel, 20 µL of reuterin was inserted into each well. Electrophoresis was performed with 50 volts at 80 mA for 2 h.

Biofilm formation on microtiter plates

In this study, the biofilm mass was obtained using the microtiter plate method because of its rapidity and efficiency.⁴⁰ In total, 20 µL of the *S. mutans* suspension, the turbidity of which was measured at OD₆₆₀: 1.0 (1.0 × 10⁸ CFU/mL bacterial concentration), was inoculated into 180 µL of BHI broth in 96-well flat bottom

microtiter plates (Greiner Bio-One, Frickenhausen, Germany) and incubated anaerobically for 24 h.

Effect of reuterin extract on biofilm formation

After the *S. mutans* suspension was incubated in anaerobic conditions for 24 h, 20 µL of the *V. parvula* suspension, the turbidity of which was measured at OD₆₆₀: 1.0 (1.0 × 10⁸ CFU/mL bacterial concentration), was inoculated into the medium in polystyrene 96-well flat bottom microtiter plates containing the same medium. The co-cultures were then incubated anaerobically for 48 h to perform the mixed biofilms containing *S. mutans* and *V. parvula*. Subsequently, the bacterial suspensions were treated with concentration stages (6.25%, 12.5%, 25%, 50%, and 100%) of reuterin extract, and the biofilm inhibitory effect was observed periodically after 15 min, 3 h, 6 h, and 24 h in the polystyrene microplate assay. Chlorhexidine at 1.2% was used as a positive control. The wells containing the biofilms without reuterin were used as negative controls. The spectrometric optical density (OD) was 490 nm, and it was used to calculate the biofilm mass. The measurement of biofilm reduction was calculated as a percentage from the blank, control, and treated absorbance values on the plate as follows:⁴¹

$$\left[\frac{(C - B) - (T - B)}{C - B} \right] \times 100\%$$

Here, B indicates the average absorbance per well for blank wells (no biofilm, no treatment), C indicates the average absorbance per well for negative control wells (biofilm, no treatment), and T indicates the average absorbance per well in treated wells (biofilm, treatment). The assays were performed in triplicate.

Statistical analysis

This experiment was performed in triplicate. The means and standard errors of the results were calculated. Statistical significance was determined by using one-way analysis of variance (ANOVA). A *p*-value less than 0.05 (*p* < 0.05) was considered statistically significant.

Data were analyzed using SPSS Statistics 24.0 software for Macintosh (IBM, Armonk, NY, USA).

Results

The SDS-PAGE results showed that the 52 kDa band visualized on the agar confirmed the presence of reuterin (Figure 1). Figure 2 showed the dot-blot results of reuterin against anti-reuterin antibodies from the mouse model, as well as the reuterin isolated from *L. reuteri* LC382415 with the anti-reuterin primary antibody from *L. reuteri* ATCC 55730, to confirm the reuterin isolated from *L. reuteri*. The SDS-PAGE results of serum mice antibodies after being treated with the reuterin results profile show that the thickest bands can be observed at 52 kDa (Figure 3).

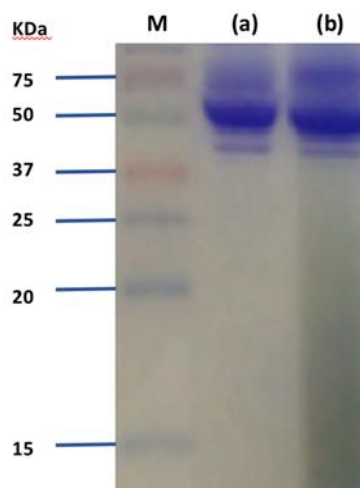


Figure 1. The SDS-PAGE results showed visualized reuterin in polyacrilamide 12% gel. M = marker protein molecular weight: 15–100 kDa. Ribbon line (a)&(b): reuterin isolate *L. reuteri* showed 52 kDa.

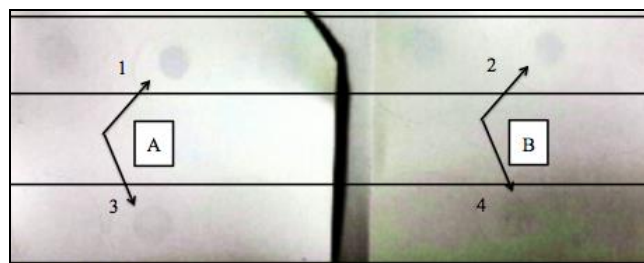


Figure 2. A. Dot-blot reuterin complex protein with glycerol dehydratase *L. reuteri* LC382415 isolate with an anti-reuterin primary antibody from *L. reuteri* ATCC 55730 (1: 250) and secondary antibodies (Ig G anti-mouse antibodies) using

adjuvants; B. Dot-blot reuterin complex protein with glycerol dehydratase *L. reuteri* ATCC 55730 isolate with an antireuterine primary antibody from *L. reuteri* ATCC 55730 (1: 250) and secondary antibodies (IgG anti-mouse antibodies) without adjuvants. Numbers 1 and 2 are reuterin antigens isolated from *L. reuteri* LC382415; 3 and 4 are reuterin antigens isolated from *L. reuteri* ATCC 55730.

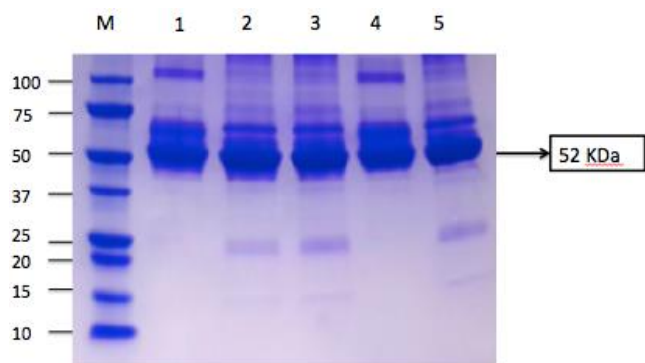


Figure 3. Visualization of SDS-PAGE. The SDS-PAGE results of serum obtained from mice are seen in the molecular weight of 52 kDa (M = marker, 1–5 = serum antibodies from mice after injection of reuterin complex protein produced with glycerol dehydratase).

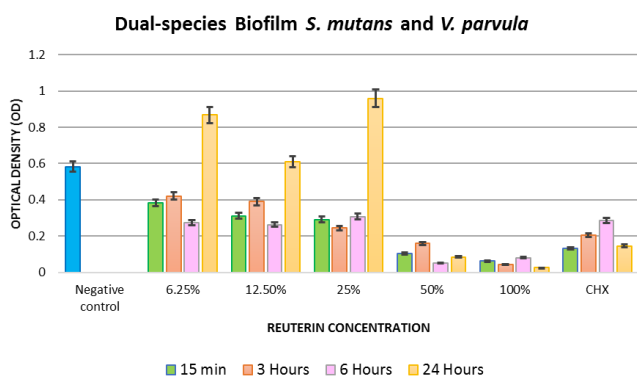


Figure 4. The inhibitory effect of reuterin extract on the dual-species biofilm *S. mutans* and *V. parvula*. Chlorhexidine at 1.2% was used as a positive control, and wells containing the biofilms without reuterin treatment were used as negative controls.

The results of the effects of reuterin on the bacterial viability of *S. mutans* and *V. parvula* are shown in Figure 4. The number of viable dual-species bacteria in the experimental group was significantly lower than that of the negative control. The number of dual species decreased in

mean OD \pm SD (OD = 0.049 \pm 0.029) after treatment with the 50% concentration of reuterin extract for 6 h. However, reuterin had the strongest activity on dual-species biofilm, with a 100% concentration of reuterin for a 24-h period (OD = 0.025 \pm 0.024). The concentration-response curves of reuterin treatment (Figure 5) show that the dual-species disinfection was significantly increased ($p < 0.05$) in concentration by reaching a 91% reduction after treatment with a 50% concentration of reuterin for 6 h.

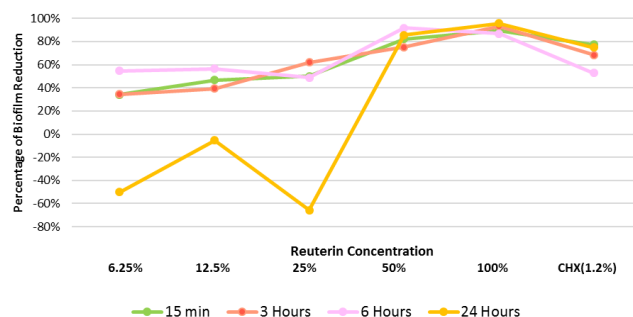


Figure 5. Concentration response curves for treatment with reuterin extract. The percentage reduction in dual-species biofilm *S. mutans* and *V. parvula* after treatment with different concentrations of reuterin extract.

Discussion

L. reuteri is an obligatory heterofermentative bacterium that produces organic acids (including lactic and acetic acid), ethanol, and carbon dioxide as its primary metabolites due to glucose metabolism.⁴² *L. reuteri* has been proven effective against oral pathogens, and its efficacy in the reduction of dental plaque and gingivitis cases among patients is undeniably significant.⁴³ Recent studies showed that probiotic consumption can reduce oral pathogens in saliva.^{44,45} Most *L. reuteri* strains can produce reuterin (3-HPA), which is a broad-range antimicrobial compound excreted by *L. reuteri* in the presence of glycerol at a certain period,²⁷ solely catalyzed by the glycerol dehydratase enzyme.⁴⁶

Reuterin has a broad spectrum of antimicrobial effects, including both gram-positive and gram-negative bacteria, fungi, and protozoa. Structurally, reuterin contains the highly reactive aldehyde group, which could lead to protein inactivation in many microorganisms.³¹ Although the precise antimicrobial activity of reuterin is not fully understood, the mechanism could be related

to the inhibition of some specific enzymes that are required in the DNA synthesis of microorganisms.⁴⁷

We found that there was an inhibitory effect of reuterin extract on the dual-species biofilm formation of *S. mutans* and *V. parvula* (Figure 4 and 5). The optimum concentration of reuterin extract for the viability of dual-species bacteria is a 100% concentration for a 24-h period. This inhibitory effect was found to be similar to that identified in another study,³⁵ where an *L. reuteri* strain was used to inhibit *S. mutans* biofilm formation *in vitro*. Because *L. reuteri* strains produce antimicrobial components, including H₂O₂, organic acids, and reuterin, the acid products of *L. reuteri* have been proven to inhibit the growth of *S. mutans* completely.³⁵

Kara et al. (2006) reported that the viability of the dual-species biofilm of *S. mutans* and *V. parvula* was higher than that of single-species biofilm. This might indicate a signal molecule transaction between *S. mutans* and *V. parvula*. This is significant because these species have the ability to produce, tolerate, or utilize lactic acid, which is important in biofilm formation.^{48,49} *Streptococcus* sp. produces lactic acid as a metabolic end product, while *Veillonella* sp. can utilize lactic acid as a nutrient for growth. The interaction at the metabolic level has been demonstrated *in vitro* and *in vivo* in gnotobiotic rats.¹³ Regardless, *Veillonella alcalescens* was used in that study instead of *V. parvula*.

The cariogenic bacteria increased acid production by producing lactic acid, which is known as the predominant metabolic end product in the presence of high sugar concentrations. Therefore, lactic acid is considered the major acidic end product in the mechanism of dental caries.⁵⁰ Based on one report, the concentration of lactic acid might remain constant over time in the dual-species biofilm caused by *V. parvula*, which may lead to a less cariogenic potential compared to the single-species biofilm formation of *S. mutans*.⁵

Conclusions

In conclusion, this experiment was the first to demonstrate the inhibitory effect of reuterin isolate *L. reuteri* in reducing the level of *S. mutans* and *V. parvula* dual-species biofilm. Therefore, these findings show that the reuterin isolate *L. reuteri* might be implicated to prevent

biofilm formation at an early stage. Further studies are needed to explore this result with other oral pathogens.

Acknowledgements

The authors are grateful to Aradhea Monica Drestia, SSi and Stella Pranoto, SSi for their laboratory assistance at the MiCORE laboratory, Faculty of Dentistry, Trisakti University. The authors also would like to thank the Faculty of Dentistry of Trisakti University for funding this study.

Declaration of Interest

We declare that there are no conflicts of interest in regard to this work.

References

1. Chen M, Yu Q, Sun H. Novel Strategies for the Prevention and Treatment of Biofilm Related Infections. *Int J Mol Sci*. 2013;14(9):18488-18501.
2. Bjarnsholt T, Alhede M, Alhede M, et al. The *in vivo* biofilm. *Trends Microbiol*. 2013;21(9):466-474.
3. Paster BJ, Boches SK, Galvin JL, et al. Bacterial Diversity in Human Subgingival Plaque. *J Bacteriol*. 2001;183(12):3770-3783.
4. Mancl KA, Kirsner RS, Ajdic D. Wound biofilms: lessons learned from oral biofilms. *Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc*. 2013;21(3):352-362.
5. Kara D, Luppens SBI, Cate JM. Differences between single- and dual-species biofilms of *Streptococcus mutans* and *Veillonella parvula* in growth, acidogenicity and susceptibility to chlorhexidine. *Eur J Oral Sci*. 2006;114(1):58-63.
6. Diaz PI, Chalmers NI, Rickard AH, et al. Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Appl Environ Microbiol*. 2006;72(4):2837-2848.
7. Nyvad B, Kilian M. Microbiology of the early colonization of human enamel and root surfaces *in vivo*. *Scand J Dent Res*. 1987;95(5):369-380.
8. Kolenbrander PE, Andersen RN, Blehert DS, Eglund PG, Foster JS, Palmer RJ. Communication among oral bacteria. *Microbiol Mol Biol Rev MMBR*. 2002;66(3):486-505.
9. Gibbons RJ, Fitzgerald RJ. Dextran-induced Agglutination of *Streptococcus mutans*, and Its Potential Role in the Formation of Microbial Dental Plaques. *J Bacteriol*. 1969;98(2):341-346.
10. McBride BC, Van der Hoeven JS. Role of interbacterial adherence in colonization of the oral cavities of gnotobiotic rats infected with *Streptococcus mutans* and *Veillonella alcalescens*. *Infect Immun*. 1981;33(2):467-472.
11. Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. *Proc Natl Acad Sci U S A*. 1999;96(25):14547-14552.
12. Moore WE, Moore LV. The bacteria of periodontal diseases. *Periodontol* 2000. 1994;5:66-77.
13. Mashima I, Nakazawa F. Interaction between *Streptococcus* spp. and *Veillonella tobetsuensis* in the Early Stages of Oral Biofilm Formation. *J Bacteriol*. 2015;197(13):2104-2111.
14. Periasamy S, Kolenbrander PE. Central Role of the Early Colonizer *Veillonella* sp. in Establishing Multispecies Biofilm Communities with Initial, Middle, and Late Colonizers of Enamel. *J Bacteriol*. 2010;192(12):2965-2972.

15. Mashima I, Theodorea CF, Thaweboon B, Thaweboon S, Nakazawa F. Identification of Veillonella Species in the Tongue Biofilm by Using a Novel One-Step Polymerase Chain Reaction Method. *PloS One*. 2016;11(6):e0157516.
16. Kanasi E, Dewhirst FE, Chalmers NI, et al. Clonal Analysis of the Microbiota of Severe Early Childhood Caries. *Caries Res*. 2010;44(5):485-497.
17. Theodorea CF, Mashima I, Thaweboon B, Thaweboon S, Nakazawa F. Molecular Detection of Oral Veillonella Species in the Saliva of Children with Different Oral Hygiene Statuses. 2017:13.
18. Sundqvist G. Associations between microbial species in dental root canal infections. *Oral Microbiol Immunol*. 1992;7(5):257-262.
19. Khemaleelakul S, Baumgartner JC, Pruksakorn S. Identification of bacteria in acute endodontic infections and their antimicrobial susceptibility. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2002;94(6):746-755.
20. Baumgartner JC, Falkler WA. Bacteria in the apical 5 mm of infected root canals. *J Endod*. 1991;17(8):380-383.
21. Peters LB, Wesselink PR, Buijs JF, van Winkelhoff AJ. Viable bacteria in root dentinal tubules of teeth with apical periodontitis. *J Endod*. 2001;27(2):76-81.
22. Delwiche EA, Pestka JJ, Tortorello ML. The veillonellae: gram-negative cocci with a unique physiology. *Annu Rev Microbiol*. 1985;39:175-193.
23. Sutter VL. Anaerobes as normal oral flora. *Rev Infect Dis*. 1984;6 Suppl 1:S62-66.
24. Chalmers NI, Palmer RJ, Cisar JO, Kolenbrander PE. Characterization of a Streptococcus sp.-Veillonella sp. community micromanipulated from dental plaque. *J Bacteriol*. 2008;190(24):8145-8154.
25. Allaker RP, Ian Douglas C. Non-conventional therapeutics for oral infections. *Virulence*. 2015;6(3):196-207.
26. Devine DA, Marsh PD. Prospects for the development of probiotics and prebiotics for oral applications. *J Oral Microbiol*. 2009;1.
27. Morelli L, Capurso L. FAO/WHO Guidelines on Probiotics: 10 Years Later. *J Clin Gastroenterol*. 2012;46:S1.
28. Mahasneh SA, Mahasneh AM. Probiotics: A Promising Role in Dental Health. *Dent J*. 2017;5(4):26.
29. Jones SE, Versalovic J. Probiotic Lactobacillus reuteribiofilms produce antimicrobial and anti-inflammatory factors. *BMC Microbiol*. 2009;9:35.
30. Cleusix V, Lacroix C, Vollenweider S, Le Blay G. Glycerol induces reuterin production and decreases Escherichia coli population in an in vitro model of colonic fermentation with immobilized human feces. *FEMS Microbiol Ecol*. 2008;63(1):56-64.
31. Vollenweider S, Lacroix C. 3-hydroxypropionaldehyde: applications and perspectives of biotechnological production. *Appl Microbiol Biotechnol*. 2004;64(1):16-27.
32. Caglar E, Cildir SK, Ergeneli S, Sandalli N, Twetman S. Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium Lactobacillus reuteri ATCC 55730 by straws or tablets. *Acta Odontol Scand*. 2006;64(5):314-318.
33. Nikawa H, Makihiro S, Fukushima H, et al. Lactobacillus reuteri in bovine milk fermented decreases the oral carriage of mutans streptococci. *Int J Food Microbiol*. 2004;95(2):219-223.
34. Iniesta M, Herrera D, Montero E, et al. Probiotic effects of orally administered Lactobacillus reuteri-containing tablets on the subgingival and salivary microbiota in patients with gingivitis. A randomized clinical trial. *J Clin Periodontol*. 2012;39(8):736-744.
35. Kang M-S, Oh J-S, Lee H-C, et al. Inhibitory effect of Lactobacillus reuteri on periodontopathic and cariogenic bacteria. *J Microbiol Seoul Korea*. 2011;49(2):193-199.
36. Widyanman AS, Pranoto S, Theodorea CF, Bachtiar EW, Bachtiar BM. Isolation and Identification of Indonesian Lactobacillus reuteri strain from Saliva of Young Adults. *Sci Dent J*. 2018;2(2):69-77.
37. Chen C-N, Liang H-F, Lin M-H, Sung H-W. A Natural Sterilant (Reuterin) Fermented from Glycerol Using Lactobacillus Reuteri: Fermentation Conditions. *J Med Biol Eng*. 2001;21(4):7.
38. Costas M. The Analysis of Bacterial Proteins by SDS Polyacrylamide Gel Electrophoresis. In: *Diagnostic Bacteriology Protocols. Methods in Molecular BiologyTM*. Humana Press; 1995:27-40.
39. Renner SW. Immunoblotting and dot immunobinding. Emerging techniques in protein immunochemistry. *Arch Pathol Lab Med*. 1988;112(8):780-786.
40. Lebeaux D, Chauhan A, Rendueles O, Beloin C. From in vitro to in vivo Models of Bacterial Biofilm-Related Infections. *Pathog Basel Switz*. 2013;2(2):288-356.
41. Pitts B, Hamilton MA, Zilver N, Stewart PS. A microtiter-plate screening method for biofilm disinfection and removal. *J Microbiol Methods*. 2003;54(2):269-276.
42. Årsköld E, Lohmeier-Vogel E, Cao R, Roos S, Rådström P, Niel EWJ van. Phosphoketolase Pathway Dominates in Lactobacillus reuteri ATCC 55730 Containing Dual Pathways for Glycolysis. *J Bacteriol*. 2008;190(1):206-212.
43. Krasse P, Carlsson B, Dahl C, Paulsson A, Nilsson A, Sinkiewicz G. Decreased gum bleeding and reduced gingivitis by the probiotic Lactobacillus reuteri. *Swed Dent J*. 2006;30(2):55-60.
44. Widyanman AS, Yunita ST, Prasetyadi T. Consumption of Yogurt Containing Probiotic Bifidobacterium Lactis Reduces Streptococcus mutans in Orthodontic Patients. *Sci Dent J*. 2018;2(1):19-25.
45. Widyanman AS, Hartono V, Marjani L, Irawan D, Luthfi L, Bachtiar BM. Lactobacillus reuteri containing Probiotic Lozenges Consumption Reduces Streptococcus mutans, Streptococcus sobrinus, Porphyromonas gingivalis, and Aggregatibacter actinomycetemcomitans in Orthodontic Patients. *J Int Dent Med Res*. 11(2):628-633.
46. Schaefer L, Auchtung TA, Hermans KE, Whitehead D, Borhan B, Britton RA. The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups. *Microbiol Read Engl*. 2010;156(Pt 6):1589-1599.
47. Talarico TL, Dobrogosz WJ. Purification and Characterization of Glycerol Dehydratase from Lactobacillus reuteri. *Appl Environ Microbiol*. 1990;56(4):1195-1197.
48. van Houte J. Role of micro-organisms in caries etiology. *J Dent Res*. 1994;73(3):672-681.
49. Filoche SK, Anderson SA, Sissons CH. Biofilm growth of Lactobacillus species is promoted by Actinomyces species and Streptococcus mutans. *Oral Microbiol Immunol*. 2004;19(5):322-326.
50. Georgios A, Vassiliki T, Sotirios K. Acidogenicity and acidurance of dental plaque and saliva sediment from adults in relation to caries activity and chlorhexidine exposure. *J Oral Microbiol*. 2015;7:26197.