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The Effect of *Lactobacillus reuteri* Probiotic Consumption on the Expression of the Various Virulence Gene Transcription Factors in Dental Plaque from Patients using Fixed Orthodontic Appliance

Abstract

Aims & Background: In orthodontic treatment, a device namely fixed orthodontic appliance is used to correct malocclusion in the patient's jaw. Patients with fixed orthodontic appliance experience difficulties in maintaining oral hygiene due to the brackets and wires of the device, which facilitate microorganism colonization and biofilm formation. *Lactobacillus reuteri* is a probiotic that can induce broad-spectrum antimicrobial substance and inhibit DNA synthesis in a pathogenic environment. By producing high concentrations of substances, such as acids and bacteriocins (antimicrobial agents) that inhibit the growth of bacteria and fungi, *L. reuteri* creates an environment unsuitable for Candida growth. The aim of this study was to determine the effect of consuming lozenges containing *L. reuteri* probiotic *L. reuteri* Prodentis lozenges on the expression of *BCR1, ACE2, fadA, aid1, gelE*, and *atlA* genes in dental biofilms of subjects using fixed orthodontic appliances.

Materials and Methods: Plaque samples (n = 20) obtained in a previous study were used in this research. Each subject consumed *L. reuteri* probiotic lozenges (2 × 108 CFU/ml) each day for 2 weeks. RNA was extracted from the samples and synthesized into cDNA. The expression of the gene transcription factors *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE* and *atlA* genes in biofilms of subjects who used fixed orthodontic appliances was detected using RT-qPCR (Real Time Quantitative Polymerase Chain Reaction)

Results: The expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes were decreased after consuming the L. reuteri probiotic lozenges for 2 weeks.

Conclusion: Consuming *L. reuteri* probiotic lozenges would affect the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* in plaque from patients using fixed orthodontic appliances.

Clinical significance: Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression, hence, helping increasing oral health of consumer.

Keywords:

biofilm, gene expression, *Lactobacillus reuteri*, orthodontic, probiotic

Introduction:

Orthodontic treatment is common today in the community. Among adults and children, orthodontic treatment may be undertaken for dental care or esthetic reasons.[1] Orthodontic treatment using fixed appliances aims to ensure proper occlusion and esthetic function, with appropriate tooth movement. Fixed orthodontic treatment can lead to changes in the oral environment and oral flora composition and an increase in the amount of plaque due to difficulty in maintaining oral hygiene.[2-5] In addition, excess composite around the base of the bracket that used in orthodontic treatment is an important factor that can cause plaque accumulation due to the presence of rough surfaces and cracks on the enamel composite surfaces.[3–5] Biofilm accumulation on teeth and soft tissues in the oral cavity can lead to caries, gingivitis, and periodontitis.[6,7]

Fusobacterium nucleatum is the dominant bacterial species and plays an important role in the formation of dental biofilms and periodontal tissue disease. In the formation of biofilms, *F. nucleatum* being a "bridging" or "linking" organism between initial bacterial colonization and final bacterial colonization, which are unable to bind to each other directly. *F. nucleatum* can also co-aggregate with various microbial species in the oral cavity.[8–10] *F. nucleatum* encodes several adhesion genes involved in interspecies interactions, including fusobacterium adhesion A (*fadA*), fusobacterial outer membrane protein A (*fomA*), *radD* (an arginine-inhibitable adhesin), and adherence inducing determinant gene 1 (*aid1*).[8,11–14] Fusobacterium adhesin A (FadA) is known to be involved in *F*. nucleatum invasion and adhesion to host cells and is highly conserved among oral Fusobacterium species.[8,15,16] FadA has been identified has a major virulence factor in *F. nucleatum* in interspecies interactions with Streptococcus mediated by *radD,* as it increases the binding specificity of *F. nucleatum* to other microbial species.[14] The arginine-inhibitable adhesion *radD* is required by *F. nucleatum* for co-adherence with various species of gram-positive bacteria, such as streptococci (early colonizers), and fungal species, such as Candida.[11,12,17]

Enterococcus faecalis (*E. faecalis*) is associated with chronic periodontitis and chronic apical periodontitis in failed root canal treatment.[18] *E. faecalis* is a gram-positive aerobic bacterium. The severity of *E. faecalis* infection depends on the immune response and virulence factors, which can exacerbate infection and play a role in increasing biofilm formation.[19] There are several genes associated with *E. faecalis* biofilm formation, including gelatinase (*gelE*) and autolysin (*atlA)*.[20] GelE in *E. faecalis* plaque or saliva isolates showed resistance to antibiotics and high biofilm formation ability.[21] atlA is the main peptidoglycan hydrolase or autolysin of *E. faecalis.*[22] AtlA plays a role in the biofilm maturation stage during which extracellular DNA (eDNA) is released and contributes to biofilm attachment and stability.[23,24]

An increase in the number of colonies of microorganisms also increased, one of which was *Candida albicans*, which causes infections of oral mucosa.[25,26] *C. albicans* has a protein in the form of an adhesive that mediates other microorganisms

to adhere to abiotic and host surfaces to form biofilms.[27] Several *C. albicans* gene transcription factors, including biofilm and cell wall regulator 1 (BCR1) and angiotensin converting enzyme 2 (ACE2), play a role in the formation of biofilms. BCR1 acts as a major regulator of *C. albicans* biofilm formation.[28] The ACE2 transcription factor plays a role in fungal adherence, biofilm formation, and hyphal morphogenesis. In addition, ACE2 plays a role in regulating the expression of genes involved in cell wall separation and metabolism.[29] As shown in previous research, ACE2 is required for filamentation, and it can increase the number of pseudohyphae cells at the time of biofilm formation.[30]

Biofilm formation plays a role in increasing antibiotic resistance in bacterial cells. Therefore, an effective therapy is needed to prevent biofilm formation. The use of probiotics has been suggested as a promising approach to prevent and treat microbial diseases and biofilm activity in the oral cavity.[31,32] Several studies have proven that the use of probiotics has oral cavity health benefits, such as preventing caries and periodontal disease.[32,33] One commercial probiotic proven to be beneficial for oral health is *Lactobacillus reuteri* (*L. reuteri*). The antimicrobial activity of *L. reuteri* inhibits colonization by pathogenic microbes and interacts the inhibition directly with host cells.[34] *L. reuteri* also inhibits the growth of *C. albicans* and *E. faecalis* biofilms.[35,36] No studies have investigated the effect of the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in fixed orthodontic appliances biofilms. Thus, the aim of this study was to determine the effect of consuming lozenges containing the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes in biofilms from subjects using fixed orthodontic appliances.

Methods:

Sample Collection

This research comprised an experimental laboratory test. Plaque samples obtained in previous study from 20 subjects who used fixed orthodontic appliances were used in this study. All the subjects had consumed *L. reuteri* probiotic lozenges (2 × 108 CFU/ml) once a day for 2 weeks prior to sample collection. Plaque samples were obtained from the subjects before and after consuming the probiotic.

RNA Extraction, cDNA Synthesis, and cDNA quantification

RNA was extracted using TRIzol reagent methodology (Thermo Fisher, Waltham, MA). The extracted RNA was synthesized into cDNA using ReverTra AceTM qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The cDNA was then quantified using an Invitrogen™ Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). The cDNA was stored at -20°C for storage of directly used for downstream analysis.

qPCR Analysis

Amplification and detection by qPCR (Applied Biosystems, Waltham, MA) were performed. The components of the qPCR Master Mix are listed in Table 2. Using a

specific kit, namely HOT FIREpol EvaGreen® qPCR Mix (Solis Biodyne, Tartu, Estonia) which was activated by incubation at 95°C for 10 minutes. This was followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing temperature at 60−65°C (Table 3), and elongation at 72°C for 20 seconds. For templates longer than 150 bp, the annealing and elongation times were extended to 30 seconds. Actin gene encoding as the housekeeping gene was used for normalization purposes. qPCR was performed on cDNA. qPCR was performed using the primers listed in Table 3.

Data Analysis

The data were analysed using the Shapiro−Wilk normality test ($p > 0.05$). For data with a normal distribution, a paired *t*-test was applied. ($p < 0.05$).

Results:

Based on the results of the qPCR test, the expression of *BCR1* (Figs. 1 and 2), *ACE2* (Figs. 3 and 4), *fadA* (Figs. 5, 6), *aid1* (Figs. 7, 8), *gelE* (Figs. 9, 10), and *atlA* (Figs. 11, 12) decreased after the subjects consumed the probiotic *L. reuteri* for 2 weeks.

As shown by the results of the Shapiro−Wilk normality test, all the data were normally distributed ($p > 0.05$). The data were analyzed using a paired *t*-test, with a significance level of $p < 0.05$. The results of the paired *t*-test revealed a significant difference in the comparison of the *BCR1* and *ACE2* gene expression data. As shown in Table 4, there was a significant difference ($p < 0.05$) in the expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes after consuming the probiotic *L. reuteri*.

Discussion:

The use of fixed orthodontic appliances often causes poor oral hygiene, thus facilitating microorganism accumulation and various pathological conditions in the oral cavity, such as fungal infections.[37] A previous study revealed an increase in Candida in saliva after using fixed orthodontic appliances. The authors attributed this to the design of fixed orthodontic appliances, which creates a space for the retention of food waste.[38] Thus, patients must be instructed about good oral hygiene practices after orthodontic treatment. [38] Fixed orthodontic appliances also induce changes in buffer capacity, salivary flow rates, and acidity (pH), leading to plaque accumulation and an increase in caries and periodontal disease.[39–41]

Biofilm formation is an important virulence factor of *F. nucleatum* due to its higher resistance to host defence or antibacterial agents compared to planktonic cells.[42] Several studies detected increased numbers of *Porphyromonas gingivalis, F. nucleatum, P. intermedia,* and *Tannerella forsythia* after the use of fixed orthodontic appliances.[15,43,44] They also reported that *F. nucleatum* increased the risk of periodontitis in orthodontic patients due to a conducive environment for anaerobic bacteria.[15,43,44] Biofilm formation is also a contributing factor to *E. faecalis* colonization and infection. Biofilms develop through various processes by which bacteria adhere to surfaces, decompose complex matrices, and develop into bacterial colonies, which adhere to surfaces.[45]

In this study, we used the qPCR method and 2-CT formula to calculate target gene expression. As shown by the results, the probiotic *L. reuteri* affected the expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes and the formation of *C.*

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albicans, F. nucleatum, and *E. faecalis* biofilms. As shown in Figure 2 and 4, the expression of *X* genes decreased in association with biofilm formation. Based on the Shapiro−Wilk normality test, all the data were normally distributed, with $p > 0.05$. The paired *t*-test results revealed significant differences in the expression *of BCR1, ACE2, fadA*, *aid1*, *gelE*, and *atlA* genes (all $p < 0.05$, Table 4).

Based on the results of this study, the probiotic *L. reuteri* significantly downregulated the transcription of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes. In gene expression, the process of translating genetic information in the form of sequence of bases of DNA or RNA into proteins.[46] Through gene expression measurement, it is possible to assess qualitatively and quantitatively the effect of a treatment, such as the administration of a drug compound.[47] Gene expression in microorganisms is involved in regulating cell-cell communication, carbohydrate metabolism, adherence, and adaptation to the surrounding environment. A decrease in the expression of specific genes can reduce microorganism colonization and microorganism numbers.[48]

BCR1, a major gene transcription factor, produces an adhesin protein, which facilitates *C. albicans* attachment to mucosal surfaces, which is a critical stage of infection.[49] Deletion of *BCR1* eliminates *C. albicans* gene function, resulting in a decrease in biofilm formation.[50] This was supported by the results of the present study, which revealed a statistically significant decrease in *BCR1* gene expression after consuming the probiotic.

The expression of *ACE2* also decreased based on the results of the statistical tests. With deletion of *ACE2*, *C. albicans* is unable to form hyphal cells, and thus biofilm formation is inhibited.[30] An earlier in vitro study showed that probiotics have antifungal effects against *C. albicans* in the oral cavity. Regular use of probiotics helped to inhibit Candida biofilms and reduced Candida colonization in the oral cavity, thereby reducing the possibility of candidiasis infection.[51]

FadA protein is the main *F. nucleatum* virulence factor and mediates microbial attachment and colonization.[8] Based on the results of this study, the probiotic *L. reuteri* appears to influence the pathogenicity of *F. nucleatum* adhesion molecules and colonization and affect biofilm formation through decreased expression of the *fadA* gene.[35] Various *F. nucleatum* adhesins mediate adhesion and aggregation and function as coaggregation intermediaries in the formation and maturation of dental biofilms.[52] The interaction of Fusobacterium with other species is largely mediated by the adhesin genes *radD* and *aid1*.[53] The *aid1* gene plays a role in interspecies interactions, colonization, and aggregation of *F. nucleatum*. In a previous study, inactivation of the *aid1* gene decreased the ability of *F. nucleatum* to coaggregate, especially with Streptococcus spp. or *E. faecalis*.[14] As shown in earlier studies, probiotics can affect the expression of genes involved in cell adhesion, quorum sensing (QS), virulence factors, and biofilm formation.[54,55]

The *E. faecalis gelE* gene has the ability to hydrolyze gelatin, collagen, fibrin, and other peptides.[56] Gene *gelE* is a virulence factor in infection formation through bacterial attachment and biofilm formation.[22] In *E. faecalis*, biofilm formation is regulated by QS, where Fsr regulates the expression of the *gelE* gene.[57] Fsr regulates the formation of *E. faecalis* biofilms through its product *gelE* and serine proteases.[58] QS is a molecular mechanism by which bacterial cells communicate with each other via signalling molecules in biofilms. If the protease encoded by a signaling factor is

decreased, communication between bacteria and biofilm formation will be disrupted.[55] GelE can also activate *atlA*, which is responsible for eDNA release at the biofilm maturation stage.[59]

AtlA is involved in the hydrolysis of peptidoglycan, which plays an important role in separating cells division after replication.[22] AtlA plays a role in the biofilm maturation stage of *E. faecalis*, during which eDNA is released and contributes to biofilm attachment and stability, biofilm defects in primary attachment, and decreased biofilm production.[20,23] This study focused on the probiotic *L. reuteri*, which has ability to secrete antimicrobial substances and compete with oral pathogens for adhesion to mucosa. In addition, *L. reuteri* can adapt and change the pH of the surrounding environment, thereby inhibiting the growth of oral pathogens.[60] The antimicrobial substances secreted by *L. reuteri* are reuterin and reutericycline.[61] Reuterin and reutericycline are broad-spectrum antimicrobial agents that are effective against gram-positive and negative bacteria, fungi, and protozoa by inhibiting microbial DNA synthesis.[35,39]

Many dental and oral health care products used daily now include probiotics. The use of probiotics is increasing due to their advantages over chemical agents, namely reducing the risk of antibiotic resistance.[62] Probiotics work by modulating the immune system, producing antimicrobial substances, and inhibiting certain pathogenic organisms by interfering with adhesion, colonization, and biofilm formation. They inhibit the growth of pathogens via the production of various substances, such as lactic acid and acetic acid, which penetrate the bacterial cell membrane and lower the cytoplasmic pH of pathogenic bacteria. Hydrogen peroxide and bacteriocin can destroy the cell membrane of pathogenic bacteria and inhibit the synthesis of pathogenic DNA.[32,33,35,63] Based on the results of this study, 2 weeks of daily consumption of the probiotic *L. reuteri* affected the process of biofilm formation by downregulating the expression of *BCR1*, *ACE2, fadA, aid1, gelE,* and *atlA g*enes, which function as adherent regulators and regulators of hyphae formation in biofilm formation. Many previous studies demonstrated that the addition of probiotics to dental and oral health care products. Probiotic can reduced pathogenic microorganisms in plaque samples from patients using fixed orthodontic appliances.[39,62,64–66] Therefore, it can be stated that the probiotic *L. reuteri* has good ability as an additional treatment for dental and oral health in patients using fixed orthodontic appliances.

Conclusion:

Based on the results of this study, it can be concluded that the probiotic *L. reuteri* influences the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in biofilm formation. By reducing the expression of those genes, the probiotic *L. reuteri* can reduce biofilm formation such as dental plaque in patients using fixed orthodontic appliances.

Clinical significance: Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression, hence, helping increasing oral health of consumer

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List of abbreviations:

^{o}C	: Celcius degree
ACE2	: Angiotensin Converting Enzyme 2
aid1	: adherence inducing determinant gene 1
atlA	: autolysin
BCR1	: Biofilm and Cell wall Regulator 1
cDNA	: copy Deoxyribonucleic Acid
CFU/mL	: Colony forming unit per mililiter
DNA	: Deoxyribonucleic Acid
eDNA	: extracellular Deoxyribonucleic Acid
fadA	: fusobacterium adhesion A
gelE	: gelatinase
radD	: arginine-inhibitable adhesin
RT-qPCR	: Quantitative Polymerase Chain Reaction

References:

1. Sihotang BM, Panjaitan M, Sitanggang JM, Putri A, Harahap FR. Perbedaan oral hygiene pemakaian pesawat ortodonti cekat dan pesawat ortodonti lepasan pada pasien klinik spesialis RS Herna. Jurnal Prima Medika Sains. 2020 Mar 15;2(1):1–5.
2. Gkantidis N, Christou P, Topouzelis N. The orthodontic-periodontic interrelationship in integrated treatment challenges: a systematic review. J Oral Rehabil. 2010 May 1;37(5):377–90.
3. Oktaviani V, Santoso O. Perbedaan Indeks Higiene Oral dan pH Plak Kelompok Pemakai dan Bukan Pemakai Pesawat Ortodonti Cekat. 2016;5(1):8.
4. Bowen WH, Burne RA, Wu H, Koo H. Oral Biofilms: Pathogens, Matrix and Polymicrobial Interactions in Microenvironments. Trends Microbiol. 2018 Mar;26(3):229–42.
5. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence. 2011;2(5):435–44.
6. Verrusio C, Iorio-Siciliano V, Blasi A, Leuci S, Adamo D, Nicolò M. The effect of orthodontic treatment on periodontal tissue inflammation: A systematic review. Quintessence Int. 2018;49(1):69–77.
7. Perkowski K, Baltaza W, Conn DB, Marczyńska-Stolarek M, Chomicz L. Examination of oral biofilm microbiota in patients using fixed orthodontic appliances in order to prevent risk factors for health complications. Ann Agric Environ Med. 2019 Jun 17;26(2):231–5.
8. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. Curr Opin Microbiol. 2015 Feb;0:141–7.
9. Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS. Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol. 2003 Feb;11(2):94–100.
10. Thurnheer T, Karygianni L, Flury M, Belibasakis GN. Fusobacterium Species and Subspecies Differentially Affect the Composition and Architecture of Supra- and Subgingival Biofilms Models. Front Microbiol. 2019 Jul 30;10:1716.
11. Chew J, Zilm PS, Fuss JM, Gully NJ. A proteomic investigation of Fusobacterium nucleatum alkaline-induced biofilms. BMC Microbiology. 2012 Sep 3;12(1):189.
12. Kaplan CW, Lux R, Haake SK, Shi W. The Fusobacterium nucleatum Outer Membrane Protein RadD Is an Arginine-Inhibitable Adhesin Required for Inter-Species Adherence and the Structured Architecture of Multi-Species Biofilm. Mol Microbiol. 2009 Jan;71(1):35–47.
13. Cochrane K, Robinson AV, Holt RA, Allen-Vercoe E. A survey of Fusobacterium nucleatum genes modulated by host cell infection. Microb Genom. 2020 Feb;6(2).

JAYPEE

14. Kaplan A, Kaplan CW, He X, McHardy I, Shi W, Lux R. Characterization of aid1, a novel gene involved in Fusobacterium nucleatum interspecies interactions. Microb Ecol. 2014 Aug;68(2):379–87.

15. Liu P, Liu Y, Wang J, Guo Y, Zhang Y, Xiao S. Detection of Fusobacterium Nucleatum and fadA Adhesin Gene in Patients with Orthodontic Gingivitis and Non-Orthodontic Periodontal Inflammation. PLOS ONE. 2014 Jan 9;9(1):e85280.

16. Brennan CA, Garrett WS. Fusobacterium nucleatum - symbiont, opportunist and oncobacterium. Nat Rev Microbiol. 2019 Mar;17(3):156–66.

17. Wu T, Cen L, Kaplan C, Zhou X, Lux R, Shi W, et al. Cellular Components Mediating Coadherence of Candida albicans and Fusobacterium nucleatum. J Dent Res. 2015 Oct;94(10):1432–8.

18. Wang Q-Q, Zhang C-F, Chu C-H, Zhu X-F. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. Int J Oral Sci. 2012 Apr;4(1):19–23.

19. Najafi K, Gholizadeh P, Tanomand A, Ahangarzadeh Rezaee M, Mahmood S, Asgharzadeh M, et al. Oral Cavity infection by Enterococcus Faecalis: virulence factors and pathogenesis. Reviews in Medical Microbiology. 2019 Jan 2;30.

20. Ch'ng J-H, Chong KKL, Lam LN, Wong JJ, Kline KA. Biofilm-associated infection by enterococci. Nat Rev Microbiol. 2019 Jan;17(2):82–94.

21. Anderson AC, Jonas D, Huber I, Karygianni L, Wölber J, Hellwig E, et al. Enterococcus faecalis from Food, Clinical Specimens, and Oral Sites: Prevalence of Virulence Factors in Association with Biofilm Formation. Front Microbiol. 2016 Jan 11;6:1534.

22. Stinemetz EK, Gao P, Pinkston KL, Montealegre MC, Murray BE, Harvey BR. Processing of the major autolysin of E. faecalis, AtlA, by the zinc-metalloprotease, GelE, impacts AtlA septal localization and cell separation. PLOS ONE. 2017 Oct 19;12(10):e0186706.

23. Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, et al. Contribution of Autolysin and Sortase A during Enterococcus faecalis DNA-Dependent Biofilm Development. Infect Immun. 2009 Sep;77(9):3626–38.

24. Paganelli FL, Willems RJL, Jansen P, Hendrickx A, Zhang X, Bonten MJM, et al. Enterococcus faecium Biofilm Formation: Identification of Major Autolysin AtlAEfm, Associated Acm Surface Localization, and AtlAEfm-Independent Extracellular DNA Release. mBio. 2013 Apr 16;4(2):e00154-13.

25. Putri MH, Herijulianti E. Ilmu Pencegahan Penyakit Jaringan Keras dan Jaringan Pendukung Gigi | OPAC Integrasi | Online Public Access Catalog | Universitas Gadjah Mada. RGC; 2010.

26. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev. 2007;20(1):133–63.

JAYPEE

27. Garcia MC, Lee JT, Ramsook CB, Alsteens D, Dufrêne YF, Lipke PN. A Role for Amyloid in Cell Aggregation and Biofilm Formation. PLOS ONE. 2011;6(3):e17632.

28. Nobile CJ, Mitchell AP. Regulation of Cell-Surface Genes and Biofilm Formation by the C. albicans Transcription Factor Bcr1p. Current Biology. 2005 Jun 21;15(12):1150–5.

29. Mulhern SM, Logue ME, Butler G. Candida albicans Transcription Factor Ace2 Regulates Metabolism and Is Required for Filamentation in Hypoxic Conditions. Eukaryot Cell. 2006 Dec;5(12):2001–13.

30. Saputo S, Kumar A, Krysan DJ. Efg1 Directly Regulates ACE2 Expression To Mediate Cross Talk between the cAMP/PKA and RAM Pathways during Candida albicans Morphogenesis. Eukaryot Cell. 2014 Sep;13(9):1169–80.

31. Widyarman AS, Bachtiar EW, Bachtiar BM, Seneviratne CJ. Inhibitory effect of probiotic lactobacilli against Streptococcus mutans and Porphyromonas gingivalis biofilms. Scientific Dental Journal. 2019 May 1;3(2):50.

32. Widyarman AS, Ramajayanti T, Theodorea CF. Indonesian Strain of Probiotic Reduces the Initial Biofilm Colonization. The Open Dentistry Journal [Internet]. 2020 Nov 13 [cited 2022 Feb 13];14(1). Available from: https://opendentistryjournal.com/VOLUME/14/PAGE/544/FULLTEXT/

33. Widyarman AS, Theodorea CF. Novel Indigenous Probiotic Lactobacillus reuteri Strain Produces Anti-biofilm Reuterin against Pathogenic Periodontal Bacteria. Eur J Dent [Internet]. 2021 Jul 24 [cited 2022 Feb 13]; Available from: http://www.thieme-connect.de/DOI/DOI?10.1055/s-0041-1731591

34. Mu Q, Tavella VJ, Luo XM. Role of Lactobacillus reuteri in Human Health and Diseases. Frontiers in Microbiology [Internet]. 2018 [cited 2022 Feb 13];9. Available from: https://www.frontiersin.org/article/10.3389/fmicb.2018.00757

35. Department of Microbiology, Faculty of Dentistry, Trisakti University, Jakarta, 11440 Indonesia, Sari Widyarman A, Kiky Elysia Lazaroni N, Undergraduate Program, Faculty of Dentistry, Trisakti University, Jakarta, 11440 Indonesia. Persistent Endodontics Pathogens Biofilm Inhibited by Lactobacillus reuteri Indonesian Strain Lactobacillus reuteri Indonesian. J Dent Indones [Internet]. 2019 Dec 31 [cited 2022 Feb 13];26(3). Available from: https://scholarhub.ui.ac.id/jdi/vol26/iss3/7/

36. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol. 2008;226(5):1065–73.

37. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. J Oral Microbiol [Internet]. 2011;3. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3087208/

38. Khanpayeh E, Jafari AA, Tabatabaei Z. Comparison of salivary Candida profile in patients with fixed and removable orthodontic appliances therapy. Iran J Microbiol. 2014 Aug;6(4):263–8.

JAYPEE

39. Widyarman AS, Hartono V, Marjani LI, 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. Journal of International Dental and Medical Research. 2018 Jan 1;11(2):628–33.

40. Restrepo M, Bussaneli DG, Jeremias F, Cordeiro RCL, Raveli DB, Magalhães AC, et al. Control of White Spot Lesions with Use of Fluoride Varnish or Chlorhexidine Gel During Orthodontic Treatment A Randomized Clinical Trial. J Clin Pediatr Dent. 2016;40(4):274–80.

41. Lu H, Tang H, Zhou T, Kang N. Assessment of the periodontal health status in patients undergoing orthodontic treatment with fixed appliances and Invisalign system. Medicine (Baltimore). 2018 Mar 30;97(13):e0248.

42. Fitriasari N, Wahyuni IS. POTENSI PROBIOTIK DALAM TATALAKSANA ORAL CANDIDIASIS: Ulasan Sistematik. ODONTO : Dental Journal. 2021 Jul 12;8(1):34–44.

43. Guo L, Feng Y, Guo H-G, Liu B-W, Zhang Y. Consequences of orthodontic treatment in malocclusion patients: clinical and microbial effects in adults and children. BMC Oral Health. 2016 Oct 28;16:112.

44. Alqerban A. Efficacy of antimicrobial photodynamic and photobiomodulation therapy against Treponema denticola, fusobacterium nucleatum and human beta defensin-2 levels in patients with gingivitis undergoing fixed orthodontic treatment: A clinic-laboratory study. Photodiagnosis and Photodynamic Therapy. 2020 Mar 1;29:101659.

45. Willett JLE, Ji MM, Dunny GM. Exploiting biofilm phenotypes for functional characterization of hypothetical genes in Enterococcus faecalis. npj Biofilms Microbiomes. 2019 Sep 19;5(1):1–14.

46. Sinaga E. Regulasi Ekspresi Gen. Regulasi Ekspresi Gen. 2011;1–24.

47. Van Peer G, Mestdagh P, Vandesompele J. Accurate RT-qPCR gene expression analysis on cell culture lysates. Sci Rep. 2012 Jan 13;2:222.

48. Shemesh M, Tam A, Steinberg D 2007. Expression of biofilm-associated genes of Streptococcus mutans in response to glucose and sucrose. Journal of Medical Microbiology. 56(11):1528–35.

49. Nikoomanesh F, Department of medical mycology, Faculty of medical science, Tarbiat Modares University, Tehran, Iran, Roudbarmohammadi S, Department of medical mycology, Faculty of medical science, Tarbiat Modares University, Tehran, Iran, Roudbary M, Department of Medical Mycology and Parasitology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran, et al. Investigation of bcr1 Gene Expression in Candida albicans Isolates by RT-PCR Technique and its Impact on Biofilm Formation. iem. 2016 Jan 1;2(1):22–4.

50. Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP. Divergent Targets of Candida albicans Biofilm Regulator Bcr1 In Vitro and In Vivo. Eukaryotic Cell. 2012 Jul;11(7):896–904.

51. Matsubara VH, Bandara HMHN, Mayer MPA, Samaranayake LP. Probiotics as Antifungals in Mucosal Candidiasis. Clin Infect Dis. 2016;62(9):1143–53.

52. Li Q, Tan L, Wang H, Kou Y, Shi X, Zhang S, et al. Fusobacterium nucleatum Interaction with Pseudomonas aeruginosa Induces Biofilm-Associated Antibiotic Tolerance via Fusobacterium Adhesin A. ACS Infect Dis. 2020 Jul 10;6(7):1686–96.

53. Kaplan A. Identification and Characterization of a Novel Lipoprotein in Fusobacterium nucleatum [Internet]. UCLA; 2015 [cited 2022 Feb 13]. Available from: https://escholarship.org/uc/item/3r90n33c

54. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402–8.

55. Barzegari A, Kheyrolahzadeh K, Hosseiniyan Khatibi SM, Sharifi S, Memar MY, Zununi Vahed S. The Battle of Probiotics and Their Derivatives Against Biofilms. Infect Drug Resist. 2020 Feb 26;13:659–72.

56. Guneser MB, Eldeniz AU. The effect of gelatinase production of Enterococcus faecalis on adhesion to dentin after irrigation with various endodontic irrigants. Acta Biomater Odontol Scand. 2016 Nov 15;2(1):144–9.

57. Hashem YA, Amin HM, Essam TM, Yassin AS, Aziz RK. Biofilm formation in enterococci: genotype-phenotype correlations and inhibition by vancomycin. Sci Rep. 2017 Jul 18;7(1):5733.

58. Wang L, Dong M, Zheng J, Song Q, Yin W, Li J, et al. Relationship of biofilm formation and gelE gene expression in Enterococcus faecalis recovered from root canals in patients requiring endodontic retreatment. J Endod. 2011 May;37(5):631–6.

59. Ali L, Goraya MU, Arafat Y, Ajmal M, Chen J-L, Yu D. Molecular Mechanism of Quorum-Sensing in Enterococcus faecalis: Its Role in Virulence and Therapeutic Approaches. Int J Mol Sci. 2017 May 3;18(5):960.

60. Bonifait L. Probiotics for Oral Health: Myth or Reality? 2009;75(8):6.

61. Urbańska M, Szajewska H. The efficacy of Lactobacillus reuteri DSM 17938 in infants and children: a review of the current evidence. Eur J Pediatr. 2014;173(10):1327–37.

62. Benic GZ, Farella M, Morgan XC, Viswam J, Heng NC, Cannon RD, et al. Oral probiotics reduce halitosis in patients wearing orthodontic braces: a randomized, triple-blind, placebo-controlled trial. J Breath Res. 2019 May 31;13(3):036010.

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63. Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A. Probiotic mechanisms of action. Ann Nutr Metab. 2012;61(2):160–74.

64. Widyarman AS, Yunita ST, Prasetyadi T. Consumption of Yogurt Containing Probiotic Bifidobacterium Lactis Reduces Streptococcus mutans in Orthodontic Patients. Scientific Dental Journal. 2018 Jan 25;2(1):19–25.

65. Alp S, Baka ZM. Effects of probiotics on salivary Streptecoccus mutans and Lactobacillus levels in orthodontic patients. Am J Orthod Dentofacial Orthop. 2018 Oct;154(4):517–23.

66. Goyal N, Shamanna PU, Varughese ST, Abraham R, Antony B, Emmatty R, et al. Effects of amine fluoride and probiotic mouthwash on levels of Porphyromonas gingivalis in orthodontic patients: A randomized controlled trial. J Indian Soc Periodontol. 2019;23(4):339–44.

67. Ranjith K, Kalyana Chakravarthy S, Adicherla H, Sharma S, Shivaji S. Temporal Expression of Genes in Biofilm-Forming Ocular *Candida albicans* Isolated From Patients With Keratitis and Orbital Cellulitis. Investigative Ophthalmology & Visual Science. 2018 Jan 25;59(1):528–38.

68. Nailis H, Coenye T, Van Nieuwerburgh F, Deforce D, Nelis HJ. Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. BMC Mol Biol. 2006 Aug 4;7:25.

69. Vos M, Quince C, Pijl AS, Hollander M de, Kowalchuk GA. A Comparison of rpoB and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity. PLOS ONE. 2012 Feb 15;7(2):e30600.

70. Zheng J-X, Wu Y, Lin Z-W, Pu Z-Y, Yao W-M, Chen Z, et al. Characteristics of and Virulence Factors Associated with Biofilm Formation in Clinical Enterococcus faecalis Isolates in China. Front Microbiol. 2017 Nov 24;8:2338.

Figures and figure legends

Figure 1. (**a**) A graph showing bcr1 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of bcr1 gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 2. (**a**) A graph showing ace2 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of ace2 gene expression in plaque samples as assessed by the RT-qPCR method and

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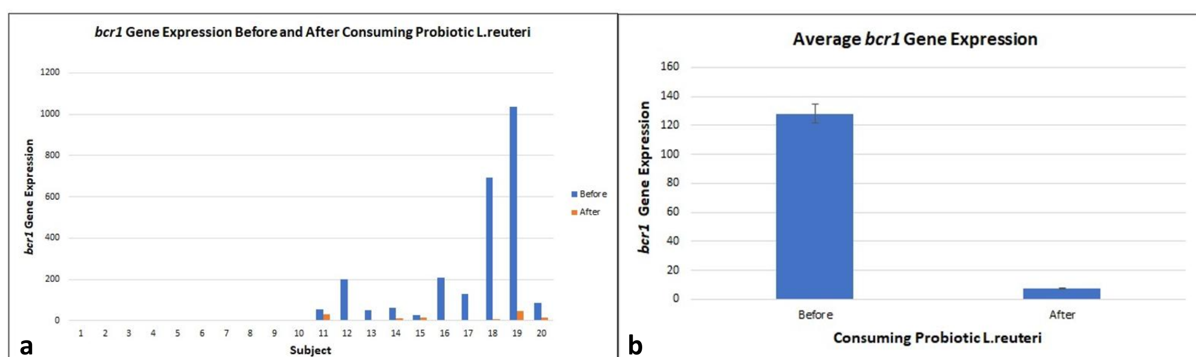
the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 3. (**a**) A graph showing fadA gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of fadA gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 4. (**a**) A graph showing aid1 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of aid1 gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 5. (**a**) A graph showing gelE gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of gelE gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 6. (**a**) A graph showing atlA gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of atlA gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges



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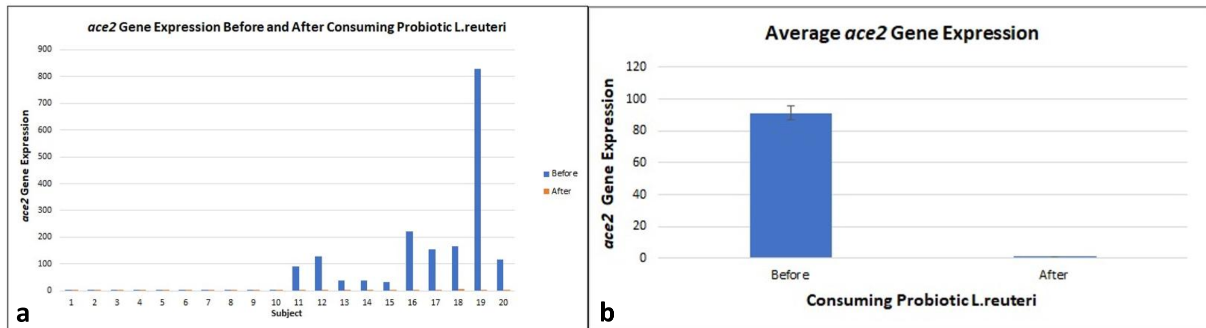

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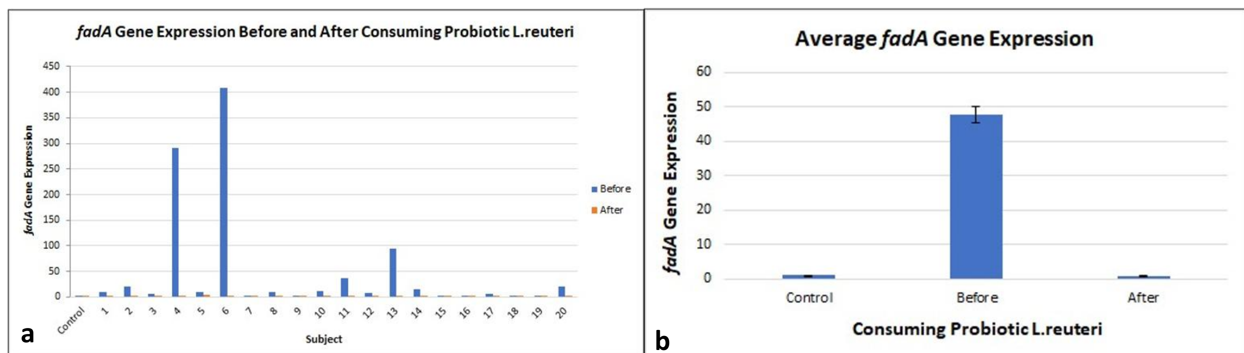

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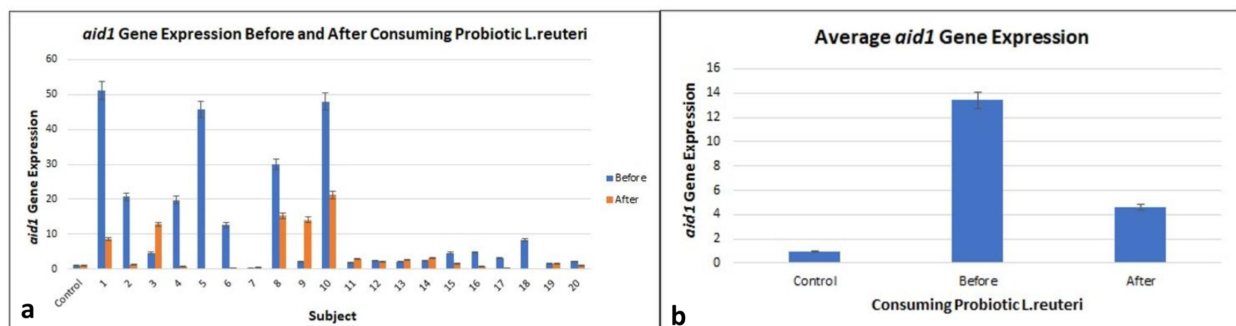

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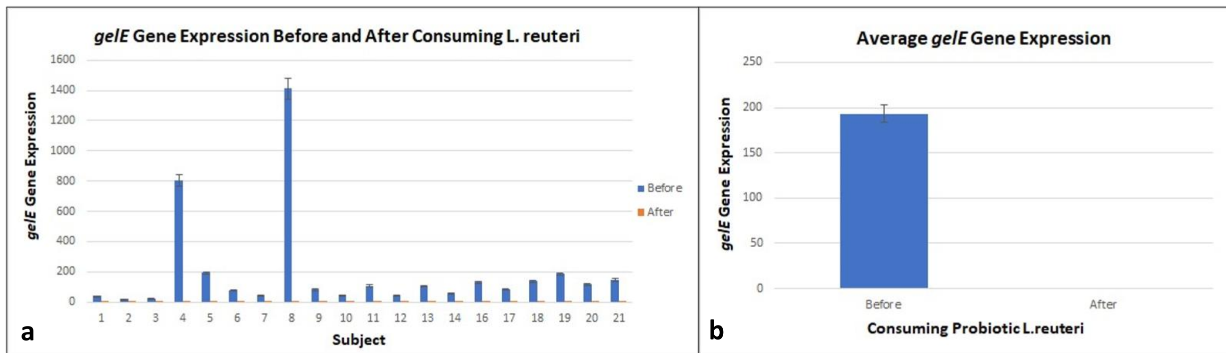

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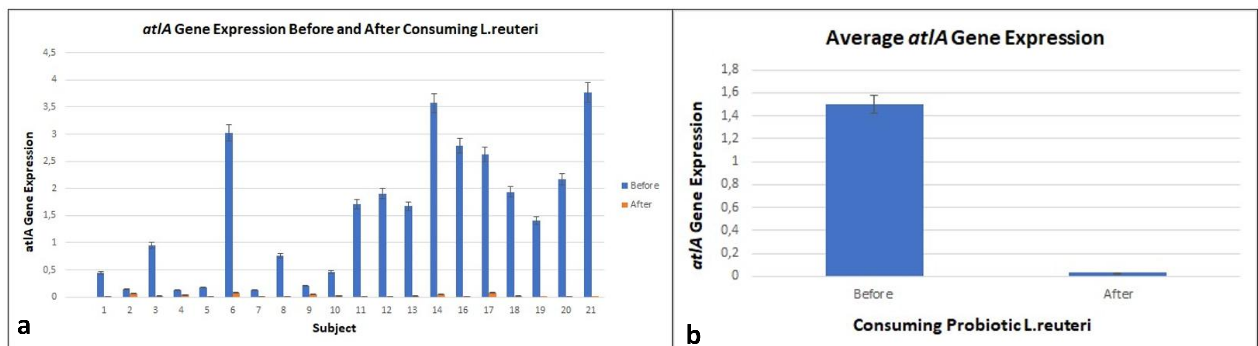

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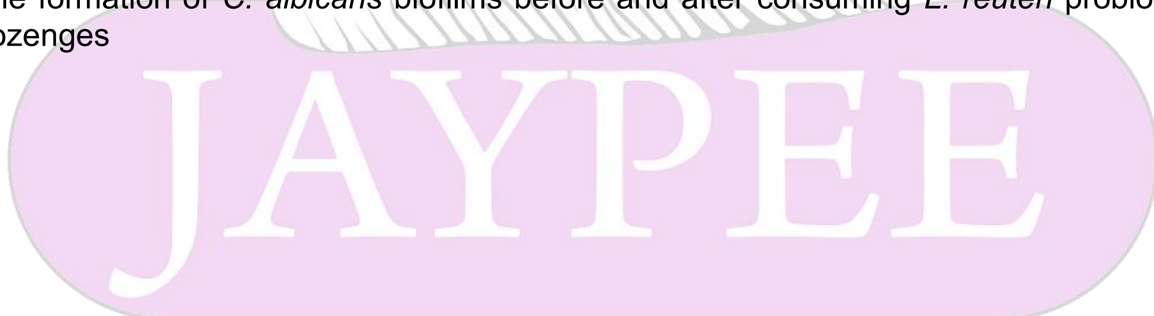

Tables and table legends

Table 1. Reagent components of the DNase I reaction solution.

Table 2. Components of the Master Mix RT-qPCR.

Table 3. Primer sequence for RT-qPCR

Table 1. Reagent components for DNase I Reaction Solution

Component	**Volume**
4x DN *Master Mix*	2 mL
RNA *template*	0,5 pg – 0,5 mg
Nuclease-free Water	X mL
Total Volume	**8 mL**

Table 2. Components of Master Mix RT-qPCR

Component	**Volume**
5 x HOT FIREPol EvaGreen® qPCR Mix Plus	4 mL
Primer *Forward*	1 mL
Primer *Reverse*	1 mL
DNA *template*	2 mL

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NFW	12 mL
Total	**20 mL**

Table 3. *Primer sequence* for RT-qPCR

Genes	***Primer sequence***	
BCR1[49]	*forward*: 5'-CTTCAGCAGCTTCATTAACACCTA-3' *reverse*: 5'-TCTTGGATCAGGTGTACTTTTCAA-3'	Initial denaturation of $95^{o}C$ for 5 minutes; 40 Cycles of denaturation at $95^{o}C$ for 1 minute and annealing at $58^{o}C$ for 1 minute.
ACE2[67]	*forward* 5'-AGAATTGACCGTTGTCCGTGTAAG-3' *reverse*: 5'-AATGGGTGAATAAATCCCTCCCTAA-3'	Initial denaturation $95^{0}C$ for 2 minutes; 40 Cycles of denaturation at $95^{o}C$ for 30 seconds and annealing at $60^{o}C$ for 1 minute.

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Housekeeping gene C. albicans : *ACT1*[68]	*forward*: 5’-TTTCATCTTCTGTATCAGAGGAACTTATTT-3’ *reverse*: 5’-ATGGGATGAATCATCAAACAAGAG-3’	Initial denaturation 95°C for 10 minutes; 40 Cycles of denaturing 95°C for 15 seconds and annealing 60°C for 1 minute
fadA[15]	*forward*: 5' -CAC AAG CTG ACG CTG CTA GA- 3' *reverse*: 5' -TTA CCA GCT CTT AAA GCT TG- 3'	Initial incubation for 4 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55.8°C for 30 seconds, and elongation at 72°C for 40 seconds and the final elongation for 6 min.[14]
aid1[14]	*forward:* 5' -TACAGGAG GTGCCGTAGCAG-3' *reverse:* 5' -TTTTTGTTAATTCT CCAGCTCCA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.[13]

Housekeeping gene *F. nucleatum*: *rpoB*[69]	*forward:* 5'- GGYTWYGAAGTNCGHGACGTDCA - 3' *reverse:* 5'-TGACGYTGCATGTTBGMR CCCATMA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.
gelE[58]	*forward:* 5'- CGGAACATACTGCCGGTTTAGA - 3' *reverse:* 5'- TGGATTAGATGCACCCGAAAT - 3'	Initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 5 seconds, and annealing at 60°C for 30 seconds.
atlA[70]	*forward:* 5'- AATAATCAATCAGGAACGAATACG - 3' *reverse:* 5'- GCCACACTAACACCGAAT -3'	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.
Housekeeping gene E. faecalis: *rpoA*[70]	*forward:* 5'- GTGAAACCTGGTCGTGGCTA - 3' *reverse:*	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.

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5’- CGACGAACGGGTGTGTAGAT-
3’

2. Bukti permintaan resubmit artikel

16 Oktober 2024

Joko Kusnoto <joko.k@trisakti.ac.id>

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The Journal The Journal of Contemporary Dental Practice requires you to resubmit your manuscript The Effect of Lactobacillus reuteri Probiotic Consumption on the Expression of the Various Virulence Gene Transcription Factors in Dental Plaque from Patients using Fixed Orthodontic Appliance in compliance to following queries.

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Dear Dr. Armelia Sari Widyarman,

The article **The Effect of Lactobacillus reuteri Probiotic Consumption on the Expression of the Various Virulence Gene Transcription Factors in Dental Plaque from Patients using Fixed Orthodontic Appliance** submitted by you through Scriptor's Zone in Journal The Journal of Contemporary Dental Practice has passed the preliminary stage and has now been further sent to Editor/Reviewers for peer-review process.

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The Effect of *Lactobacillus reuteri* Probiotic Consumption on the Expression of the Various Virulence Gene Transcription Factors in Dental Plaque from Patients using Fixed Orthodontic Appliance

Joko Kusnoto[1], Siti Sara Safirah[2], Litayana Ria Anggriani Sitorus[2], Winnie Valentini[2], Armelia Sari Widyarman[3]

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Abstract

Aims & Background: In orthodontic treatment, a device namely fixed orthodontic appliance is used to correct malocclusion in the patient's jaw. Patients with fixed orthodontic appliance experience difficulties in maintaining oral hygiene due to the brackets and wires of the device, which facilitate microorganism colonization and biofilm formation. *Lactobacillus reuteri* is a probiotic that can induce broad-spectrum antimicrobial substance and inhibit DNA synthesis in a pathogenic environment. By producing high concentrations of substances, such as acids and bacteriocins (antimicrobial agents) that inhibit the growth of bacteria and fungi, *L. reuteri* creates an environment unsuitable for Candida growth. The aim of this study was to determine the effect of consuming lozenges containing *L. reuteri* probiotic *L. reuteri* Prodentis lozenges on the expression of *BCR1, ACE2*, *fadA, aid1, gelE*, and *atlA* genes in dental biofilms of subjects using fixed orthodontic appliances.

Materials and Methods: Plaque samples (n = 20) obtained in a previous study were used in this research. Each subject consumed *L. reuteri* probiotic lozenges (2 × 108 CFU/ml) each day for 2 weeks. RNA was extracted from the samples and synthesized into cDNA. The expression of the gene transcription factors *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE* and *atlA* genes in biofilms of subjects who used fixed orthodontic appliances was detected using RT-qPCR (Real Time Quantitative Polymerase Chain Reaction)

Results: The expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes were decreased after consuming the L. reuteri probiotic lozenges for 2 weeks.

Conclusion: Consuming *L. reuteri* probiotic lozenges would affect the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* in plaque from patients using fixed orthodontic appliances.

Clinical significance: Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression, hence, helping increasing oral health of consumer.

Keywords: biofilm, gene expression, *Lactobacillus reuteri*, orthodontic, probiotic

Introduction:

Orthodontic treatment is common today in the community. Among adults and children, orthodontic treatment may be undertaken for dental care or esthetic reasons.[1] Orthodontic treatment using fixed appliances aims to ensure proper occlusion and esthetic function, with appropriate tooth movement. Fixed orthodontic treatment can lead to changes in the oral environment and oral flora composition and an increase in the amount of plaque due to difficulty in maintaining oral hygiene.[2-5] In addition, excess composite around the base of the bracket that used in orthodontic treatment is an important factor that can cause plaque accumulation due to the presence of rough surfaces and cracks on the enamel composite surfaces.[3–5] Biofilm accumulation on teeth and soft tissues in the oral cavity can lead to caries, gingivitis, and periodontitis.[6,7]

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Fusobacterium nucleatum is the dominant bacterial species and plays an important role in the formation of dental biofilms and periodontal tissue disease. In the formation of biofilms, *F. nucleatum* being a “bridging” or “linking” organism between initial bacterial colonization and final bacterial colonization, which are unable to bind to each other directly. *F. nucleatum* can also co-aggregate with various microbial species in the oral cavity.[8–10] *F. nucleatum* encodes several adhesion genes involved in interspecies interactions, including fusobacterium adhesion A (*fadA*), fusobacterial outer membrane protein A (*fomA*), *radD* (an arginine-inhibitable adhesin), and adherence inducing determinant gene 1 (*aid1*).[8,11–14] Fusobacterium adhesin A (FadA) is known to be involved in *F.* nucleatum invasion and adhesion to host cells and is highly conserved among oral Fusobacterium species.[8,15,16] FadA has been identified has a major virulence factor in *F. nucleatum* in interspecies interactions with Streptococcus mediated by *radD,* as it increases the binding specificity of *F. nucleatum* to other microbial species.[14] The arginine-inhibitable adhesion *radD* is required by *F. nucleatum* for co-adherence with various species of gram-positive bacteria, such as streptococci (early colonizers), and fungal species, such as Candida.[11,12,17]

Enterococcus faecalis (*E. faecalis*) is associated with chronic periodontitis and chronic apical periodontitis in failed root canal treatment.[18] *E. faecalis* is a gram-positive aerobic bacterium. The severity of *E. faecalis* infection depends on the immune response and virulence factors, which can exacerbate infection and play a role in increasing biofilm formation.[19] There are several genes associated with *E. faecalis* biofilm formation, including gelatinase (*gelE*) and autolysin (*atlA)*.[20] GelE in *E. faecalis* plaque or saliva isolates showed resistance to antibiotics and high biofilm formation ability.[21] atlA is the main peptidoglycan hydrolase or autolysin of *E. faecalis.*[22] AtlA plays a role in the biofilm maturation stage during which extracellular DNA (eDNA) is released and contributes to biofilm attachment and stability.[23,24]

An increase in the number of colonies of microorganisms also increased, one of which was *Candida albicans*, which causes infections of oral mucosa.[25,26] *C. albicans* has a protein in the form of an adhesive that mediates other microorganisms to adhere to abiotic and host surfaces to form biofilms.[27] Several *C. albicans* gene transcription factors, including biofilm and cell wall regulator 1 (BCR1) and angiotensin converting enzyme 2 (ACE2), play a role in the formation of biofilms. BCR1 acts as a major regulator of *C. albicans* biofilm formation.[28] The ACE2 transcription factor plays a role in fungal adherence, biofilm formation, and hyphal morphogenesis. In addition, ACE2 plays a role in regulating the expression of genes involved in cell wall separation and metabolism.[29] As shown in previous research, ACE2 is required for filamentation, and it can increase the number of pseudohyphae cells at the time of biofilm formation.[30]

Biofilm formation plays a role in increasing antibiotic resistance in bacterial cells. Therefore, an effective therapy is needed to prevent biofilm formation. The use of probiotics has been suggested as a promising approach to prevent and treat microbial diseases and biofilm activity in the oral cavity.[31,32] Several studies have

proven that the use of probiotics has oral cavity health benefits, such as preventing caries and periodontal disease.[32,33] One commercial probiotic proven to be beneficial for oral health is *Lactobacillus reuteri* (*L. reuteri*). The antimicrobial activity of *L. reuteri* inhibits colonization by pathogenic microbes and interacts the inhibition directly with host cells.[34] *L. reuteri* also inhibits the growth of *C. albicans* and *E. faecalis* biofilms.[35,36] No studies have investigated the effect of the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in fixed orthodontic appliances biofilms. Thus, the aim of this study was to determine the effect of consuming lozenges containing the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes in biofilms from subjects using fixed orthodontic appliances.

Methods:

Sample Collection

This research comprised an experimental laboratory test. Plaque samples obtained in previous study from 20 subjects who used fixed orthodontic appliances were used in this study. All the subjects had consumed *L. reuteri* probiotic lozenges (2 × 108 CFU/ml) once a day for 2 weeks prior to sample collection. Plaque samples were obtained from the subjects before and after consuming the probiotic.

RNA Extraction, cDNA Synthesis, and cDNA quantification

RNA was extracted using TRIzol reagent methodology (Thermo Fisher, Waltham, MA). The extracted RNA was synthesized into cDNA using ReverTra AceTM qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The cDNA was then quantified using an Invitrogen™ Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). The cDNA was stored at -20°C for storage of directly used for downstream analysis.

qPCR Analysis

Amplification and detection by qPCR (Applied Biosystems, Waltham, MA) were performed. The components of the qPCR Master Mix are listed in Table 2. Using a specific kit, namely HOT FIREpol EvaGreen® qPCR Mix (Solis Biodyne, Tartu, Estonia) which was activated by incubation at 95°C for 10 minutes. This was followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing temperature at 60−65°C (Table 3), and elongation at 72°C for 20 seconds. For templates longer than 150 bp, the annealing and elongation times were extended to 30 seconds. Actin gene encoding as the housekeeping gene was used for normalization purposes. qPCR was performed on cDNA. qPCR was performed using the primers listed in Table 3.

Data Analysis

The data were analysed using the Shapiro−Wilk normality test ($p > 0.05$). For data with a normal distribution, a paired *t*-test was applied. ($p < 0.05$).

Results:

Based on the results of the qPCR test, the expression of *BCR1* (Figs. 1 and 2), *ACE2* (Figs. 3 and 4), *fadA* (Figs. 5, 6), *aid1* (Figs. 7, 8), *gelE* (Figs. 9, 10), and *atlA*

(Figs. 11, 12) decreased after the subjects consumed the probiotic *L. reuteri* for 2 weeks.

As shown by the results of the Shapiro−Wilk normality test, all the data were normally distributed ($p > 0.05$). The data were analyzed using a paired *t*-test, with a significance level of $p < 0.05$. The results of the paired *t*-test revealed a significant difference in the comparison of the *BCR1* and *ACE2* gene expression data. As shown in Table 4, there was a significant difference ($p < 0.05$) in the expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes after consuming the probiotic *L. reuteri*.

Discussion:

The use of fixed orthodontic appliances often causes poor oral hygiene, thus facilitating microorganism accumulation and various pathological conditions in the oral cavity, such as fungal infections.[37] A previous study revealed an increase in Candida in saliva after using fixed orthodontic appliances. The authors attributed this to the design of fixed orthodontic appliances, which creates a space for the retention of food waste.[38] Thus, patients must be instructed about good oral hygiene practices after orthodontic treatment. [38] Fixed orthodontic appliances also induce changes in buffer capacity, salivary flow rates, and acidity (pH), leading to plaque accumulation and an increase in caries and periodontal disease.[39–41]

Biofilm formation is an important virulence factor of *F. nucleatum* due to its higher resistance to host defence or antibacterial agents compared to planktonic cells.[42] Several studies detected increased numbers of *Porphyromonas gingivalis, F. nucleatum, P. intermedia,* and *Tannerella forsythia* after the use of fixed orthodontic appliances.[15,43,44] They also reported that *F. nucleatum* increased the risk of periodontitis in orthodontic patients due to a conducive environment for anaerobic bacteria.[15,43,44] Biofilm formation is also a contributing factor to *E. faecalis* colonization and infection. Biofilms develop through various processes by which bacteria adhere to surfaces, decompose complex matrices, and develop into bacterial colonies, which adhere to surfaces.[45]

In this study, we used the qPCR method and 2-CT formula to calculate target gene expression. As shown by the results, the probiotic *L. reuteri* affected the expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes and the formation of *C. albicans, F. nucleatum*, and *E. faecalis* biofilms. As shown in Figure 2 and 4, the expression of *X* genes decreased in association with biofilm formation. Based on the Shapiro−Wilk normality test, all the data were normally distributed, with $p > 0.05$. The paired *t*-test results revealed significant differences in the expression *of BCR1, ACE2, fadA*, *aid1*, *gelE*, and *atlA* genes (all $p < 0.05$, Table 4).

Based on the results of this study, the probiotic *L. reuteri* significantly downregulated the transcription of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes. In gene expression, the process of translating genetic information in the form of sequence of bases of DNA or RNA into proteins.[46] Through gene expression measurement, it is possible to assess qualitatively and quantitatively the effect of a treatment, such as the administration of a drug compound.[47] Gene expression in microorganisms is involved in regulating cell-cell communication, carbohydrate metabolism, adherence, and adaptation to the surrounding environment. A decrease in the expression of specific genes can reduce microorganism colonization and microorganism numbers.[48]

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BCR1, a major gene transcription factor, produces an adhesin protein, which facilitates *C. albicans* attachment to mucosal surfaces, which is a critical stage of infection.[49] Deletion of *BCR1* eliminates *C. albicans* gene function, resulting in a decrease in biofilm formation.[50] This was supported by the results of the present study, which revealed a statistically significant decrease in *BCR1* gene expression after consuming the probiotic.

The expression of *ACE2* also decreased based on the results of the statistical tests. With deletion of *ACE2*, *C. albicans* is unable to form hyphal cells, and thus biofilm formation is inhibited.[30] An earlier in vitro study showed that probiotics have antifungal effects against *C. albicans* in the oral cavity. Regular use of probiotics helped to inhibit Candida biofilms and reduced Candida colonization in the oral cavity, thereby reducing the possibility of candidiasis infection.[51]

FadA protein is the main *F. nucleatum* virulence factor and mediates microbial attachment and colonization.[8] Based on the results of this study, the probiotic *L. reuteri* appears to influence the pathogenicity of *F. nucleatum* adhesion molecules and colonization and affect biofilm formation through decreased expression of the *fadA* gene.[35] Various *F. nucleatum* adhesins mediate adhesion and aggregation and function as coaggregation intermediaries in the formation and maturation of dental biofilms.[52] The interaction of Fusobacterium with other species is largely mediated by the adhesin genes *radD* and *aid1*.[53] The *aid1* gene plays a role in interspecies interactions, colonization, and aggregation of *F. nucleatum*. In a previous study, inactivation of the *aid1* gene decreased the ability of *F. nucleatum* to coaggregate, especially with Streptococcus spp. or *E. faecalis*.[14] As shown in earlier studies, probiotics can affect the expression of genes involved in cell adhesion, quorum sensing (QS), virulence factors, and biofilm formation.[54,55]

The *E. faecalis gelE* gene has the ability to hydrolyze gelatin, collagen, fibrin, and other peptides.[56] Gene *gelE* is a virulence factor in infection formation through bacterial attachment and biofilm formation.[22] In *E. faecalis*, biofilm formation is regulated by QS, where Fsr regulates the expression of the *gelE* gene.[57] Fsr regulates the formation of *E. faecalis* biofilms through its product *gelE* and serine proteases.[58] QS is a molecular mechanism by which bacterial cells communicate with each other via signalling molecules in biofilms. If the protease encoded by a signaling factor is decreased, communication between bacteria and biofilm formation will be disrupted.[55] GelE can also activate *atlA*, which is responsible for eDNA release at the biofilm maturation stage.[59]

AtlA is involved in the hydrolysis of peptidoglycan, which plays an important role in separating cells division after replication.[22] AtlA plays a role in the biofilm maturation stage of *E. faecalis*, during which eDNA is released and contributes to biofilm attachment and stability, biofilm defects in primary attachment, and decreased biofilm production.[20,23] This study focused on the probiotic *L. reuteri*, which has ability to secrete antimicrobial substances and compete with oral pathogens for adhesion to mucosa. In addition, *L. reuteri* can adapt and change the pH of the surrounding environment, thereby inhibiting the growth of oral pathogens.[60] The antimicrobial substances secreted by *L. reuteri* are reuterin and reutericycline.[61] Reuterin and reutericycline are broad-spectrum antimicrobial agents that are effective against gram-positive and negative bacteria, fungi, and protozoa by inhibiting microbial DNA synthesis.[35,39]

Many dental and oral health care products used daily now include probiotics. The use of probiotics is increasing due to their advantages over chemical agents, namely reducing the risk of antibiotic resistance.[62] Probiotics work by modulating the immune system, producing antimicrobial substances, and inhibiting certain pathogenic organisms by interfering with adhesion, colonization, and biofilm formation. They inhibit the growth of pathogens via the production of various substances, such as lactic acid and acetic acid, which penetrate the bacterial cell membrane and lower the cytoplasmic pH of pathogenic bacteria. Hydrogen peroxide and bacteriocin can destroy the cell membrane of pathogenic bacteria and inhibit the synthesis of pathogenic DNA.[32,33,35,63] Based on the results of this study, 2 weeks of daily consumption of the probiotic *L. reuteri* affected the process of biofilm formation by downregulating the expression of *BCR1*, *ACE2, fadA, aid1, gelE,* and *atlA* genes, which function as adherent regulators and regulators of hyphae formation in biofilm formation. Many previous studies demonstrated that the addition of probiotics to dental and oral health care products. Probiotic can reduced pathogenic microorganisms in plaque samples from patients using fixed orthodontic appliances.[39,62,64–66] Therefore, it can be stated that the probiotic *L. reuteri* has good ability as an additional treatment for dental and oral health in patients using fixed orthodontic appliances.

Conclusion:

Based on the results of this study, it can be concluded that the probiotic *L. reuteri* influences the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in biofilm formation. By reducing the expression of those genes, the probiotic *L. reuteri* can reduce biofilm formation such as dental plaque in patients using fixed orthodontic appliances.

Clinical significance: Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression, hence, helping increasing oral health of consumer

List of abbreviations:

^{o}C : Celcius degree

ACE2 : Angiotensin Converting Enzyme 2

aid1 : adherence inducing determinant gene 1

atlA : autolysin

BCR1 : Biofilm and Cell wall Regulator 1

cDNA : copy Deoxyribonucleic Acid

CFU/mL : Colony forming unit per mililiter

DNA : Deoxyribonucleic Acid

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eDNA	: extracellular Deoxyribonucleic Acid
fadA	: fusobacterium adhesion A
gelE	: gelatinase
radD	: arginine-inhibitable adhesin
RT-qPCR	: Quantitative Polymerase Chain Reaction

References:

1. Sihotang BM, Panjaitan M, Sitanggang JM, Putri A, Harahap FR. Perbedaan oral hygiene pemakaian pesawat ortodonti cekat dan pesawat ortodonti lepasan pada pasien klinik spesialis RS Herna. Jurnal Prima Medika Sains. 2020 Mar 15;2(1):1–5.
2. Gkantidis N, Christou P, Topouzelis N. The orthodontic-periodontic interrelationship in integrated treatment challenges: a systematic review. J Oral Rehabil. 2010 May 1;37(5):377–90.
3. Oktaviani V, Santoso O. Perbedaan Indeks Higiene Oral dan pH Plak Kelompok Pemakai dan Bukan Pemakai Pesawat Ortodonti Cekat. 2016;5(1):8.
4. Bowen WH, Burne RA, Wu H, Koo H. Oral Biofilms: Pathogens, Matrix and Polymicrobial Interactions in Microenvironments. Trends Microbiol. 2018 Mar;26(3):229–42.
5. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence. 2011;2(5):435–44.
6. Verrusio C, Iorio-Siciliano V, Blasi A, Leuci S, Adamo D, Nicolò M. The effect of orthodontic treatment on periodontal tissue inflammation: A systematic review. Quintessence Int. 2018;49(1):69–77.
7. Perkowski K, Baltaza W, Conn DB, Marczyńska-Stolarek M, Chomicz L. Examination of oral biofilm microbiota in patients using fixed orthodontic appliances in order to prevent risk factors for health complications. Ann Agric Environ Med. 2019 Jun 17;26(2):231–5.
8. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. Curr Opin Microbiol. 2015 Feb;0:141–7.
9. Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS. Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol. 2003 Feb;11(2):94–100.
10. Thurnheer T, Karygianni L, Flury M, Belibasakis GN. Fusobacterium Species and Subspecies Differentially Affect the Composition and Architecture of Supra- and Subgingival Biofilms Models. Front Microbiol. 2019 Jul 30;10:1716.
11. Chew J, Zilm PS, Fuss JM, Gully NJ. A proteomic investigation of Fusobacterium nucleatum alkaline-induced biofilms. BMC Microbiology. 2012 Sep 3;12(1):189.
12. Kaplan CW, Lux R, Haake SK, Shi W. The Fusobacterium nucleatum Outer Membrane Protein RadD Is an Arginine-Inhibitable Adhesin Required for Inter-Species Adherence and the Structured Architecture of Multi-Species Biofilm. Mol Microbiol. 2009 Jan;71(1):35–47.
13. Cochrane K, Robinson AV, Holt RA, Allen-Vercoe E. A survey of Fusobacterium nucleatum genes modulated by host cell infection. Microb Genom. 2020 Feb;6(2).

JAYPEE

14. Kaplan A, Kaplan CW, He X, McHardy I, Shi W, Lux R. Characterization of aid1, a novel gene involved in Fusobacterium nucleatum interspecies interactions. Microb Ecol. 2014 Aug;68(2):379–87.

15. Liu P, Liu Y, Wang J, Guo Y, Zhang Y, Xiao S. Detection of Fusobacterium Nucleatum and fadA Adhesin Gene in Patients with Orthodontic Gingivitis and Non-Orthodontic Periodontal Inflammation. PLOS ONE. 2014 Jan 9;9(1):e85280.

16. Brennan CA, Garrett WS. Fusobacterium nucleatum - symbiont, opportunist and oncobacterium. Nat Rev Microbiol. 2019 Mar;17(3):156–66.

17. Wu T, Cen L, Kaplan C, Zhou X, Lux R, Shi W, et al. Cellular Components Mediating Coadherence of Candida albicans and Fusobacterium nucleatum. J Dent Res. 2015 Oct;94(10):1432–8.

18. Wang Q-Q, Zhang C-F, Chu C-H, Zhu X-F. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. Int J Oral Sci. 2012 Apr;4(1):19–23.

19. Najafi K, Gholizadeh P, Tanomand A, Ahangarzadeh Rezaee M, Mahmood S, Asgharzadeh M, et al. Oral Cavity infection by Enterococcus Faecalis: virulence factors and pathogenesis. Reviews in Medical Microbiology. 2019 Jan 2;30.

20. Ch'ng J-H, Chong KKL, Lam LN, Wong JJ, Kline KA. Biofilm-associated infection by enterococci. Nat Rev Microbiol. 2019 Jan;17(2):82–94.

21. Anderson AC, Jonas D, Huber I, Karygianni L, Wölber J, Hellwig E, et al. Enterococcus faecalis from Food, Clinical Specimens, and Oral Sites: Prevalence of Virulence Factors in Association with Biofilm Formation. Front Microbiol. 2016 Jan 11;6:1534.

22. Stinemetz EK, Gao P, Pinkston KL, Montealegre MC, Murray BE, Harvey BR. Processing of the major autolysin of E. faecalis, AtlA, by the zinc-metalloprotease, GelE, impacts AtlA septal localization and cell separation. PLOS ONE. 2017 Oct 19;12(10):e0186706.

23. Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, et al. Contribution of Autolysin and Sortase A during Enterococcus faecalis DNA-Dependent Biofilm Development. Infect Immun. 2009 Sep;77(9):3626–38.

24. Paganelli FL, Willems RJL, Jansen P, Hendrickx A, Zhang X, Bonten MJM, et al. Enterococcus faecium Biofilm Formation: Identification of Major Autolysin AtlAEfm, Associated Acm Surface Localization, and AtlAEfm-Independent Extracellular DNA Release. mBio. 2013 Apr 16;4(2):e00154-13.

25. Putri MH, Herijulianti E. Ilmu Pencegahan Penyakit Jaringan Keras dan Jaringan Pendukung Gigi | OPAC Integrasi | Online Public Access Catalog | Universitas Gadjah Mada. RGC; 2010.

26. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev. 2007;20(1):133–63.

27. Garcia MC, Lee JT, Ramsook CB, Alsteens D, Dufrêne YF, Lipke PN. A Role for Amyloid in Cell Aggregation and Biofilm Formation. PLOS ONE. 2011;6(3):e17632.

28. Nobile CJ, Mitchell AP. Regulation of Cell-Surface Genes and Biofilm Formation by the C. albicans Transcription Factor Bcr1p. Current Biology. 2005 Jun 21;15(12):1150–5.

29. Mulhern SM, Logue ME, Butler G. Candida albicans Transcription Factor Ace2 Regulates Metabolism and Is Required for Filamentation in Hypoxic Conditions. Eukaryot Cell. 2006 Dec;5(12):2001–13.

30. Saputo S, Kumar A, Krysan DJ. Efg1 Directly Regulates ACE2 Expression To Mediate Cross Talk between the cAMP/PKA and RAM Pathways during Candida albicans Morphogenesis. Eukaryot Cell. 2014 Sep;13(9):1169–80.

31. Widyarman AS, Bachtiar EW, Bachtiar BM, Seneviratne CJ. Inhibitory effect of probiotic lactobacilli against Streptococcus mutans and Porphyromonas gingivalis biofilms. Scientific Dental Journal. 2019 May 1;3(2):50.

32. Widyarman AS, Ramajayanti T, Theodorea CF. Indonesian Strain of Probiotic Reduces the Initial Biofilm Colonization. The Open Dentistry Journal [Internet]. 2020 Nov 13 [cited 2022 Feb 13];14(1). Available from: https://opendentistryjournal.com/VOLUME/14/PAGE/544/FULLTEXT/

33. Widyarman AS, Theodorea CF. Novel Indigenous Probiotic Lactobacillus reuteri Strain Produces Anti-biofilm Reuterin against Pathogenic Periodontal Bacteria. Eur J Dent [Internet]. 2021 Jul 24 [cited 2022 Feb 13]; Available from: http://www.thieme-connect.de/DOI/DOI?10.1055/s-0041-1731591

34. Mu Q, Tavella VJ, Luo XM. Role of Lactobacillus reuteri in Human Health and Diseases. Frontiers in Microbiology [Internet]. 2018 [cited 2022 Feb 13];9. Available from: https://www.frontiersin.org/article/10.3389/fmicb.2018.00757

35. Department of Microbiology, Faculty of Dentistry, Trisakti University, Jakarta, 11440 Indonesia, Sari Widyarman A, Kiky Elysia Lazaroni N, Undergraduate Program, Faculty of Dentistry, Trisakti University, Jakarta, 11440 Indonesia. Persistent Endodontics Pathogens Biofilm Inhibited by Lactobacillus reuteri Indonesian Strain Lactobacillus reuteri Indonesian. J Dent Indones [Internet]. 2019 Dec 31 [cited 2022 Feb 13];26(3). Available from: https://scholarhub.ui.ac.id/jdi/vol26/iss3/7/

36. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol. 2008;226(5):1065–73.

37. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. J Oral Microbiol [Internet]. 2011;3. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3087208/

38. Khanpayeh E, Jafari AA, Tabatabaei Z. Comparison of salivary Candida profile in patients with fixed and removable orthodontic appliances therapy. Iran J Microbiol. 2014 Aug;6(4):263–8.

39. Widyarman AS, Hartono V, Marjani LI, 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. Journal of International Dental and Medical Research. 2018 Jan 1;11(2):628–33.
40. Restrepo M, Bussaneli DG, Jeremias F, Cordeiro RCL, Raveli DB, Magalhães AC, et al. Control of White Spot Lesions with Use of Fluoride Varnish or Chlorhexidine Gel During Orthodontic Treatment A Randomized Clinical Trial. J Clin Pediatr Dent. 2016;40(4):274–80.
41. Lu H, Tang H, Zhou T, Kang N. Assessment of the periodontal health status in patients undergoing orthodontic treatment with fixed appliances and Invisalign system. Medicine (Baltimore). 2018 Mar 30;97(13):e0248.
42. Fitriasari N, Wahyuni IS. POTENSI PROBIOTIK DALAM TATALAKSANA ORAL CANDIDIASIS: Ulasan Sistematik. ODONTO : Dental Journal. 2021 Jul 12;8(1):34–44.
43. Guo L, Feng Y, Guo H-G, Liu B-W, Zhang Y. Consequences of orthodontic treatment in malocclusion patients: clinical and microbial effects in adults and children. BMC Oral Health. 2016 Oct 28;16:112.
44. Alqerban A. Efficacy of antimicrobial photodynamic and photobiomodulation therapy against Treponema denticola, fusobacterium nucleatum and human beta defensin-2 levels in patients with gingivitis undergoing fixed orthodontic treatment: A clinic-laboratory study. Photodiagnosis and Photodynamic Therapy. 2020 Mar 1;29:101659.
45. Willett JLE, Ji MM, Dunny GM. Exploiting biofilm phenotypes for functional characterization of hypothetical genes in Enterococcus faecalis. npj Biofilms Microbiomes. 2019 Sep 19;5(1):1–14.
46. Sinaga E. Regulasi Ekspresi Gen. Regulasi Ekspresi Gen. 2011;1–24.
47. Van Peer G, Mestdagh P, Vandesompele J. Accurate RT-qPCR gene expression analysis on cell culture lysates. Sci Rep. 2012 Jan 13;2:222.
48. Shemesh M, Tam A, Steinberg D 2007. Expression of biofilm-associated genes of Streptococcus mutans in response to glucose and sucrose. Journal of Medical Microbiology. 56(11):1528–35.
49. Nikoomanesh F, Department of medical mycology, Faculty of medical science, Tarbiat Modares University, Tehran, Iran, Roudbarmohammadi S, Department of medical mycology, Faculty of medical science, Tarbiat Modares University, Tehran, Iran, Roudbary M, Department of Medical Mycology and Parasitology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran, et al. Investigation of bcr1 Gene Expression in Candida albicans Isolates by RT-PCR Technique and its Impact on Biofilm Formation. iem. 2016 Jan 1;2(1):22–4.

50. Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP. Divergent Targets of Candida albicans Biofilm Regulator Bcr1 In Vitro and In Vivo. Eukaryotic Cell. 2012 Jul;11(7):896–904.

51. Matsubara VH, Bandara HMHN, Mayer MPA, Samaranayake LP. Probiotics as Antifungals in Mucosal Candidiasis. Clin Infect Dis. 2016;62(9):1143–53.

52. Li Q, Tan L, Wang H, Kou Y, Shi X, Zhang S, et al. Fusobacterium nucleatum Interaction with Pseudomonas aeruginosa Induces Biofilm-Associated Antibiotic Tolerance via Fusobacterium Adhesin A. ACS Infect Dis. 2020 Jul 10;6(7):1686–96.

53. Kaplan A. Identification and Characterization of a Novel Lipoprotein in Fusobacterium nucleatum [Internet]. UCLA; 2015 [cited 2022 Feb 13]. Available from: https://escholarship.org/uc/item/3r90n33c

54. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402–8.

55. Barzegari A, Kheyrolahzadeh K, Hosseiniyan Khatibi SM, Sharifi S, Memar MY, Zununi Vahed S. The Battle of Probiotics and Their Derivatives Against Biofilms. Infect Drug Resist. 2020 Feb 26;13:659–72.

56. Guneser MB, Eldeniz AU. The effect of gelatinase production of Enterococcus faecalis on adhesion to dentin after irrigation with various endodontic irrigants. Acta Biomater Odontol Scand. 2016 Nov 15;2(1):144–9.

57. Hashem YA, Amin HM, Essam TM, Yassin AS, Aziz RK. Biofilm formation in enterococci: genotype-phenotype correlations and inhibition by vancomycin. Sci Rep. 2017 Jul 18;7(1):5733.

58. Wang L, Dong M, Zheng J, Song Q, Yin W, Li J, et al. Relationship of biofilm formation and gelE gene expression in Enterococcus faecalis recovered from root canals in patients requiring endodontic retreatment. J Endod. 2011 May;37(5):631–6.

59. Ali L, Goraya MU, Arafat Y, Ajmal M, Chen J-L, Yu D. Molecular Mechanism of Quorum-Sensing in Enterococcus faecalis: Its Role in Virulence and Therapeutic Approaches. Int J Mol Sci. 2017 May 3;18(5):960.

60. Bonifait L. Probiotics for Oral Health: Myth or Reality? 2009;75(8):6.

61. Urbańska M, Szajewska H. The efficacy of Lactobacillus reuteri DSM 17938 in infants and children: a review of the current evidence. Eur J Pediatr. 2014;173(10):1327–37.

62. Benic GZ, Farella M, Morgan XC, Viswam J, Heng NC, Cannon RD, et al. Oral probiotics reduce halitosis in patients wearing orthodontic braces: a randomized, triple-blind, placebo-controlled trial. J Breath Res. 2019 May 31;13(3):036010.
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63. Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A. Probiotic mechanisms of action. Ann Nutr Metab. 2012;61(2):160–74.

64. Widyarman AS, Yunita ST, Prasetyadi T. Consumption of Yogurt Containing Probiotic Bifidobacterium Lactis Reduces Streptococcus mutans in Orthodontic Patients. Scientific Dental Journal. 2018 Jan 25;2(1):19–25.

65. Alp S, Baka ZM. Effects of probiotics on salivary Streptecoccus mutans and Lactobacillus levels in orthodontic patients. Am J Orthod Dentofacial Orthop. 2018 Oct;154(4):517–23.

66. Goyal N, Shamanna PU, Varughese ST, Abraham R, Antony B, Emmatty R, et al. Effects of amine fluoride and probiotic mouthwash on levels of Porphyromonas gingivalis in orthodontic patients: A randomized controlled trial. J Indian Soc Periodontol. 2019;23(4):339–44.

67. Ranjith K, Kalyana Chakravarthy S, Adicherla H, Sharma S, Shivaji S. Temporal Expression of Genes in Biofilm-Forming Ocular *Candida albicans* Isolated From Patients With Keratitis and Orbital Cellulitis. Investigative Ophthalmology & Visual Science. 2018 Jan 25;59(1):528–38.

68. Nailis H, Coenye T, Van Nieuwerburgh F, Deforce D, Nelis HJ. Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. BMC Mol Biol. 2006 Aug 4;7:25.

69. Vos M, Quince C, Pijl AS, Hollander M de, Kowalchuk GA. A Comparison of rpoB and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity. PLOS ONE. 2012 Feb 15;7(2):e30600.

70. Zheng J-X, Wu Y, Lin Z-W, Pu Z-Y, Yao W-M, Chen Z, et al. Characteristics of and Virulence Factors Associated with Biofilm Formation in Clinical Enterococcus faecalis Isolates in China. Front Microbiol. 2017 Nov 24;8:2338.

Figures and figure legends

Figure 1. (**a**) A graph showing bcr1 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of bcr1 gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 2. (**a**) A graph showing ace2 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of ace2 gene expression in plaque samples as assessed by the RT-qPCR method and

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the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 3. (**a**) A graph showing fadA gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of fadA gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 4. (**a**) A graph showing aid1 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of aid1 gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 5. (**a**) A graph showing gelE gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of gelE gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Figure 6. (**a**) A graph showing atlA gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of atlA gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

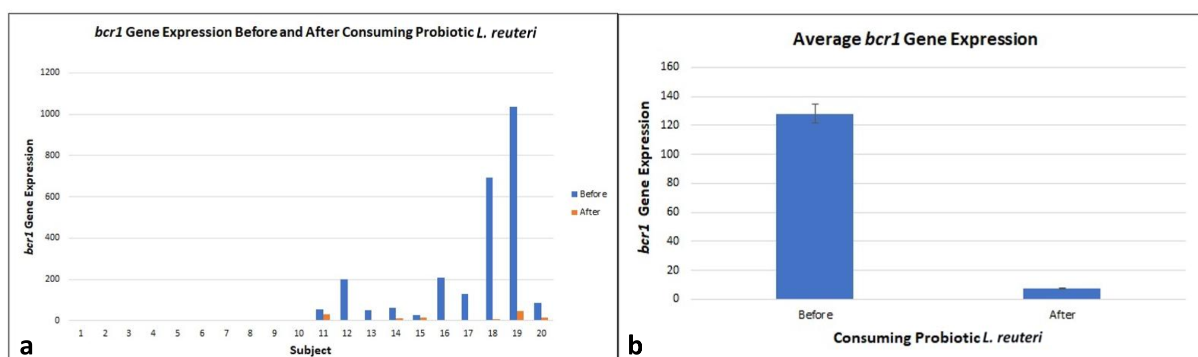

Figure 1. (**a**) A graph showing bcr1 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of bcr1 gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

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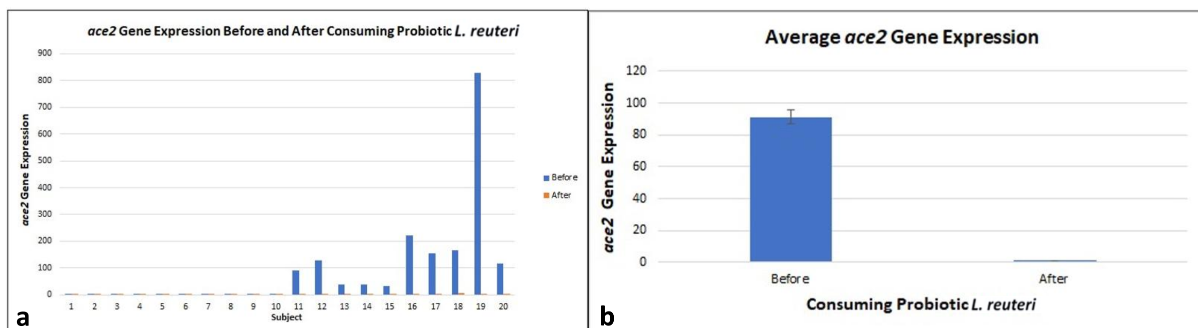


Figure 2. (**a**) A graph showing ace2 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of ace2 gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

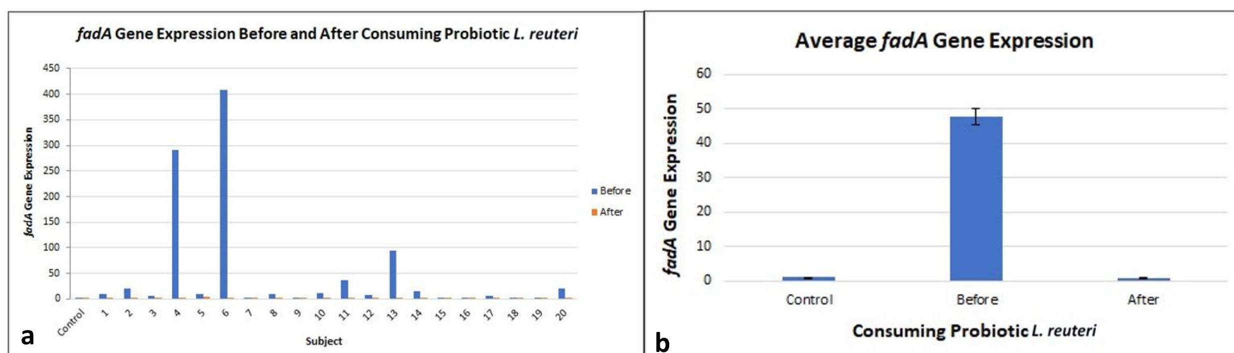


Figure 3. (**a**) A graph showing fadA gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of fadA gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

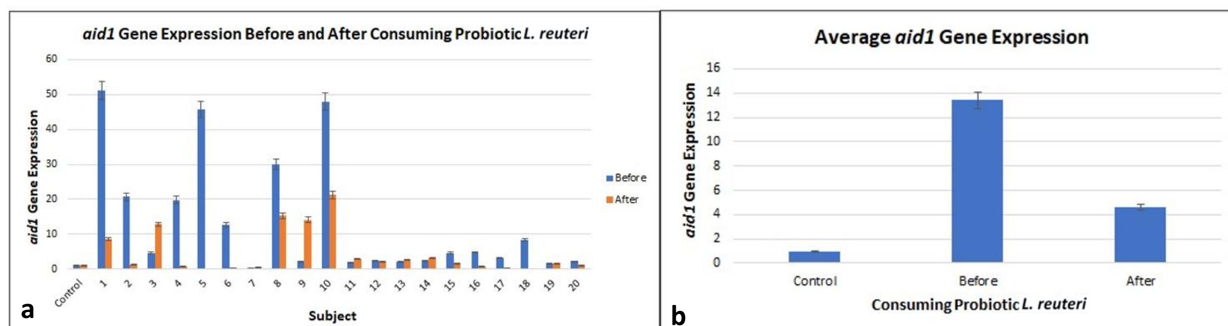


Figure 4. (**a**) A graph showing aid1 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of aid1 gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

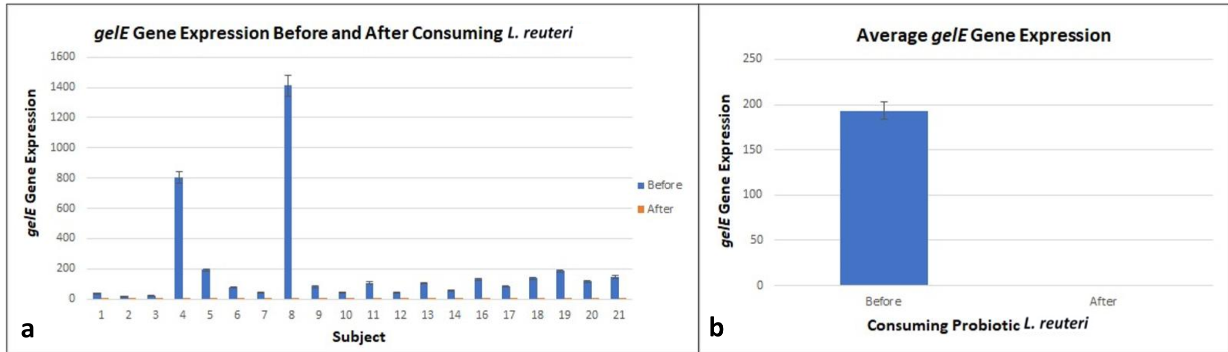


Figure 5. (**a**) A graph showing gelE gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of gelE gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

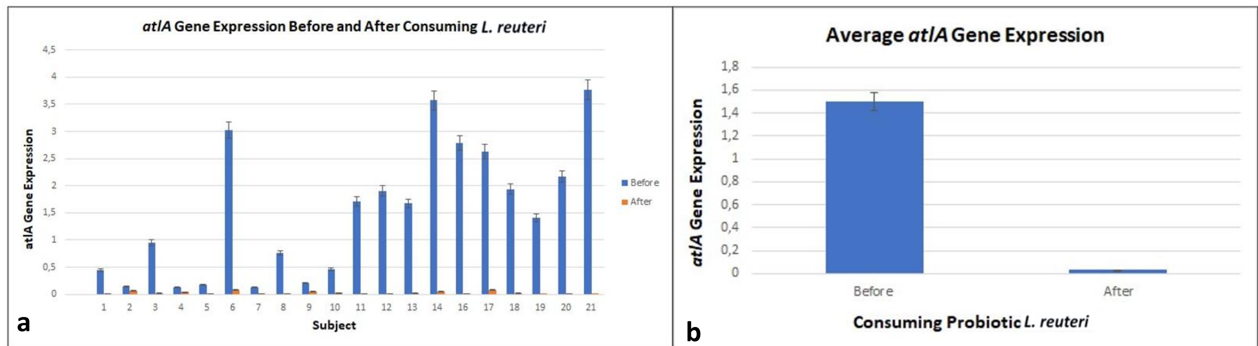
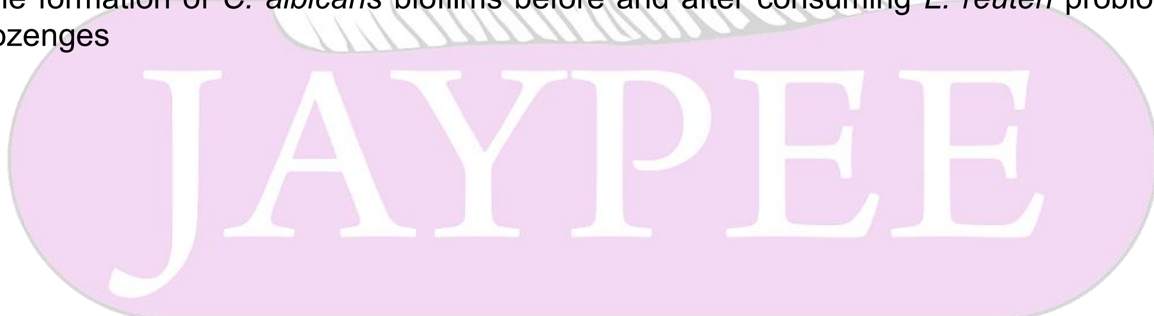


Figure 6. (**a**) A graph showing atlA gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of atlA gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges



Tables and table legends

Table 1. Reagent components of the DNase I reaction solution.

Table 2. Components of the Master Mix RT-qPCR.

Table 3. Primer sequence for RT-qPCR

Table 1. Reagent components for DNase I Reaction Solution

Component	**Volume**
4x DN *Master Mix*	2 mL
RNA *template*	0,5 pg – 0,5 mg
Nuclease-free Water	X mL
Total Volume	**8 mL**

Table 2. Components of Master Mix RT-qPCR

Component	**Volume**
5 x HOT FIREPol EvaGreen® qPCR Mix Plus	4 mL
Primer *Forward*	1 mL
Primer *Reverse*	1 mL
DNA *template*	2 mL

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NFW	12 mL
Total	**20 mL**

Table 3. *Primer sequence* for RT-qPCR

Genes	***Primer sequence***	
BCR1[49]	*forward*: 5’- CTTCAGCAGCTTCATTAACACCTA -3’ *reverse*: 5’- TCTTGGATCAGGTGTACTTTTCAA- 3’	Initial denaturation of $95^{o}C$ for 5 minutes; 40 Cycles of denaturation at $95^{o}C$ for 1 minute and annealing at $58^{o}C$ for 1 minute.
ACE2[67]	*forward* 5’- AGAATTGACCGTTGTCCGTGTAA G-3’ *reverse*: 5’- AATGGGTGAATAAATCCCTCCCTA A-3’	Initial denaturation $95^{0}C$ for 2 minutes; 40 Cycles of denaturation at $95^{o}C$ for 30 seconds and annealing at $60^{o}C$ for 1 minute.

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Housekeeping gene C. albicans : *ACT1*[68]	*forward*: 5'-TTTCATCTTCTGTATCAGAGGAACTTATTT-3' *reverse*: 5'-ATGGGATGAATCATCAAACAAGAG-3'	Initial denaturation 95°C for 10 minutes; 40 Cycles of denaturing 95°C for 15 seconds and annealing 60°C for 1 minute
fadA[15]	*forward*: 5' -CAC AAG CTG ACG CTG CTA GA- 3' *reverse*: 5' -TTA CCA GCT CTT AAA GCT TG- 3'	Initial incubation for 4 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55.8°C for 30 seconds, and elongation at 72°C for 40 seconds and the final elongation for 6 min.[14]
aid1[14]	*forward:* 5' -TACAGGAG GTGCCGTAGCAG-3' *reverse:* 5' -TTTTTGTTAATTCT CCAGCTCCA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.[13]

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Housekeeping gene *F. nucleatum*: *rpoB*[69]	*forward:* 5'- GGYTWYGAAGTNCGHGACGTDCA - 3' *reverse:* 5'-TGACGYTGCATGTTBGMR CCCATMA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.
gelE[58]	*forward:* 5’- CGGAACATACTGCCGGTTTAGA - 3’ *reverse:* 5’- TGGATTAGATGCACCCGAAAT - 3’	Initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 5 seconds, and annealing at 60°C for 30 seconds.
atlA[70]	*forward:* 5’- AATAATCAATCAGGAACGAATACG - 3’ *reverse:* 5’- GCCACACTAACACCGAAT -3’	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.
Housekeeping gene E. faecalis: rpoA[70]	*forward:* 5’- GTGAAACCTGGTCGTGGCTA - 3’ *reverse:*	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.

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5’- CGACGAACGGGTGTGTAGAT-
3’

4. Bukti konfirmasi submit revisi, respon kepada reviewer, dan artikel yang diresubmit

18 Desember 2024

Joko Kusnoto <joko.k@trisakti.ac.id>

JCDP_24_408_R2 has been sent for review

1 message

noreply@jaypeejournals.com <noreply@jaypeejournals.com> Wed, Dec 18, 2024 at 12:50 PM
To: armeliasari@trisakti.ac.id, joko.k@trisakti.ac.id, sarasafirah@gmail.com, litayanas@gmail.com, winnie.valentini@gmail.com
Cc: ayushi.goyal@jaypeebrothers.com

Dear Dr. Armelia Sari Widyarman,

The article **Lactobacillus reuteri Probiotic Consumption reduced Various Virulence Gene Expression in Dental Plaque of Fixed Orthodontic Subjects** submitted by you through Scriptor's Zone in Journal The Journal of Contemporary Dental Practice has passed the preliminary stage and has now been further sent to Editor/Reviewers for peer-review process.

Once the review is over, you will be informed of the Editorial decision via e-mail.

We thank you for your contribution to the journal and look forward for a continued support.

Thanking you

Managing Editor - Jaypee Journals
The Journal of Contemporary Dental Practice

Reviewer Number	Comments of the reviewer	Reply by the author(s)	Changes done on page number and line number
Reviewer 1	Kindly provide complete individual Author details and provide contact details of corresponding author, looks like some data is missing please confirm if data is correct and complete. Title of manuscript should be 20 words or less so please revise	Corrections have been done accordingly	Title Page
	Prepare a **short running title** for your manuscript to be used as header for each page. (Length: not more than 10 words)	We have revised accordingly	Title Page
	Abstract: change aim and background to aim only and present in a single sentence. Elaborate conclusion and clinical significance	We have done the revision as requested	Abstract Page
	Figures: provide representative gene images, rename all the bar graphs as graph 1, 2 etc and place all tables , graphs , figures at the end of the main article with legends	The revision has been done in accordance to the recommendation	Figure legend
	Explain the need to perform the study, the novelty and then finally end with a specific aim of the study.	It has been explained in the introduction	Page 4 line 139-148

	Brief about the genes assessed in the study	The introduction section has briefly explained the target genes' importance	Introduction
	Explain study design and justify sample size calculation	This is a preliminary attempt of clinical trial using the probiotic lozenges on patients with orthodontic patients. Twenty subjects were achieved also due to budget limitation and COVID-19 pandemic.	Sample collection section
	Mention approval number for IEC	We have included in the passage	Page 4 line 154-155
	Explain when the study was conducted with duration	The duration has been mentioned in the passage	Page 5 line 172-176
	Clearly explain the study groups and sample size in each group with inclusion and exclusion criteria in detail.	We have elaborated further in the sample collection section	Page 4 line 152 – page 6 line 193
	Explain the method in detail so that experiment can be repeated	Most of the downstream analysis require commercial reagents which also included the manual for using such reagents from the manufacturer. We followed the protocol per manufacturer's instruction for each reagent.	Page 6 line 195
	Outcome analysis done to be explained	We have attempted to explain this aspect	Page 6 line 219-221
	Elaborate statistical analysis used with software and version	We have included the information	Page 6 line 218-219

	Elaborate results in detail the segment is too short explain results with actual values and statistical values	The values have been included in the paper. We tried to explain the data in more simpler ways, as in averaged per gene target.	Page 7 line 225-235
	This segment should not just give legends for figures and tables. the legends to be at the end of the article	The legends have been put at the end of the article	
	First explain why this study was conducted	We have explained it in the first paragraph	Page 7 line 238-248
	Then justify the material used and technique used for outcome analysis with references	All the material used were a protocol required for assessing gene expression. RNA from plaque samples were extracted. RNA was reverse transcribed into complementary DNA (cDNA) since PCR can only detect DNA samples. Then cDNA was mixed with reagents and was run into the RT-qPCR instrument. The outcome data was calculated using $2^{-\Delta\Delta CT}$ analysis.	Page 8 line 259-265
	Compare each part of the result with similar studies and give reason if concurrent or controversial	Each gene target has been compared with its previous study. The functionality of the gene was explained followed by the implication of reduction of such gene.	Page 8 line 276-281, line 282-287, line 288 – page 9 line 300, line 301 – 310, line 311 – 322.
	Explain the limitations and future directions	We have added this information in the last paragraph of the discussion	Page 9 line 341-349

	Elaborate the message given to clinicians based on outcome of this study	We have generalized the information and included the message for clinicians.	Page 10 line 358 – 362

Lactobacillus reuteri Probiotic Consumption reduced Various Virulence Gene Expression in Dental Plaque of Fixed Orthodontic Subjects.

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57
Abstract

58 **Aims:** The aim of this study was to determine the effect of consuming lozenges
59 containing *L. reuteri* probiotic Prodentis lozenges on the expression of *BCR1, ACE2*,
60 *fadA, aid1, gelE*, and *atlA* genes in dental biofilms of subjects using fixed orthodontic
61 appliances.
62 **Materials and Methods:** Plaque samples (n = 20) obtained in a previous study were
63 used in this research. Each subject consumed *L. reuteri* probiotic lozenges (2 × 10^8
64 CFU/ml) each day for 2 weeks. RNA was extracted from the samples and synthesized
65 into cDNA. The expression of the gene transcription factors *BCR1*, *ACE2*, *fadA*, *aid1*,
66 *gelE* and *atlA* genes in biofilms of subjects who used fixed orthodontic appliances was
67 detected using RT-qPCR (Real Time Quantitative Polymerase Chain Reaction)
68 **Results:** The expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes were
69 decreased after consuming the L. reuteri probiotic lozenges for 2 weeks.
70 **Conclusion:** Consuming *L. reuteri* probiotic lozenges would affect the expression of
71 *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* in plaque from patients using fixed orthodontic
72 appliances. By reducing the expression of the virulence genes, bacterial number would
73 be reduced and biofilm production can also be reduced.
74 **Clinical significance:** Consumption of probiotic lozenges were confirmed to reduce
75 bacterial and fungal biofilm, as proven by the reduction of virulence gene expression.
76 Routine consumption of probiotic lozenges can help reduce potential bacterial
77 infection and increase the oral health of patients using fixed orthodontic appliances.
78 **Keywords:** biofilm, gene expression, *Lactobacillus reuteri*, orthodontic, probiotic
79

80
Introduction:

81 Orthodontic treatment is common today in the community. Among adults and
82 children, orthodontic treatment may be undertaken for dental care or esthetic
83 reasons.[1] Orthodontic treatment using fixed appliances aims to ensure proper
84 occlusion and esthetic function, with appropriate tooth movement. Fixed orthodontic
85 treatment can lead to changes in the oral environment and oral flora composition and
86 an increase in the amount of plaque due to difficulty in maintaining oral hygiene.[2-5] In
87 addition, excess composite around the base of the bracket that used in orthodontic
88 treatment is an important factor that can cause plaque accumulation due to the
89 presence of rough surfaces and cracks on the enamel composite surfaces.[3–5] Biofilm

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90 accumulation on teeth and soft tissues in the oral cavity can lead to caries, gingivitis,
91 and periodontitis.[6,7]

92 *Fusobacterium nucleatum* is the dominant bacterial species and plays an
93 important role in the formation of dental biofilms and periodontal tissue disease. In the
94 formation of biofilms, *F. nucleatum* being a "bridging" or "linking" organism between
95 initial bacterial colonization and final bacterial colonization, which are unable to bind
96 to each other directly. *F. nucleatum* can also co-aggregate with various microbial
97 species in the oral cavity.[8–10] *F. nucleatum* encodes several adhesion genes involved
98 in interspecies interactions, including fusobacterium adhesion A (*fadA*), fusobacterial
99 outer membrane protein A (*fomA*), *radD* (an arginine-inhibitable adhesin), and
100 adherence inducing determinant gene 1 (*aid1*).[8,11–14] Fusobacterium adhesin A (FadA)
101 is known to be involved in *F. nucleatum* invasion and adhesion to host cells and is
102 highly conserved among oral Fusobacterium species.[8,15,16] FadA has been identified
103 has a major virulence factor in *F. nucleatum* in interspecies interactions with
104 *Streptococcus* mediated by *radD,* as it increases the binding specificity of *F. nucleatum*
105 to other microbial species.[14] The arginine-inhibitable adhesion *radD* is required by *F.*
106 *nucleatum* for co-adherence with various species of gram-positive bacteria, such as
107 streptococci (early colonizers), and fungal species, such as *Candida*.[11,12,17]

108 *Enterococcus faecalis* (*E. faecalis*) is associated with chronic periodontitis and
109 chronic apical periodontitis in failed root canal treatment.[18] *E. faecalis* is a gram-
110 positive aerobic bacterium. The severity of *E. faecalis* infection depends on the
111 immune response and virulence factors, which can exacerbate infection and play a
112 role in increasing biofilm formation.[19] There are several genes associated with *E.*
113 *faecalis* biofilm formation, including gelatinase (*gelE*) and autolysin (*atlA)*.[20] GelE in *E.*
114 *faecalis* plaque or saliva isolates showed resistance to antibiotics and high biofilm
115 formation ability.[21] atlA is the main peptidoglycan hydrolase or autolysin of *E.*
116 *faecalis.*[22] AtlA plays a role in the biofilm maturation stage during which extracellular
117 DNA (eDNA) is released and contributes to biofilm attachment and stability.[23,24]

118 An increase in the number of colonies of microorganisms also increased, one
119 of which was *Candida albicans*, which causes infections of oral mucosa.[25,26] *C.*
120 *albicans* has a protein in the form of an adhesive that mediates other microorganisms
121 to adhere to abiotic and host surfaces to form biofilms.[27] Several *C. albicans* gene
122 transcription factors, including biofilm and cell wall regulator 1 (*BCR1*) and angiotensin
123 converting enzyme 2 (*ACE2*), play a role in the formation of biofilms. BCR1 acts as a

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124 major regulator of *C. albicans* biofilm formation.[28] The ACE2 transcription factor plays
125 a role in fungal adherence, biofilm formation, and hyphal morphogenesis. In addition,
126 ACE2 plays a role in regulating the expression of genes involved in cell wall separation
127 and metabolism.[29] As shown in previous research, ACE2 is required for filamentation,
128 and it can increase the number of pseudohyphae cells at the time of biofilm
129 formation.[30]

130 Biofilm formation plays a role in increasing antibiotic resistance in bacterial
131 cells. Therefore, an effective therapy is needed to prevent biofilm formation. The use
132 of probiotics has been suggested as a promising approach to prevent and treat
133 microbial diseases and biofilm activity in the oral cavity.[31,32] Several studies have
134 proven that the use of probiotics has oral cavity health benefits, such as preventing
135 caries and periodontal disease.[32,33] One commercial probiotic proven to be beneficial
136 for oral health is *Lactobacillus reuteri*. The antimicrobial activity of *L. reuteri* inhibits
137 colonization by pathogenic microbes and interacts the inhibition directly with host
138 cells.[34] *L. reuteri* also inhibits the growth of *C. albicans* and *E. faecalis* biofilms.[35,36]
139 However, no studies have investigated the effect of the probiotic *L. reuteri* on the
140 expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in dental plaque biofilms
141 found in patient's oral environment with fixed orthodontic appliances. By analyzing the
142 virulence genes found in several pathogenic bacteria, we can hypothesize that the
143 downregulation of these genes will lead to less biofilm production and healthier oral
144 health conditions during the duration of using fixed orthodontic appliances. Thus, to
145 bridge the knowledge gap, the aim of this study was to determine the effect of
146 consuming lozenges containing the probiotic *L. reuteri* on the expression of *BCR1*,
147 *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes in biofilms from subjects using fixed
148 orthodontic appliances.
149

150 ## Methods:

151 ### Sample Collection

152 Subjects undergoing fixed orthodontic therapy were enrolled in this open-label
153 prospective clinical trial. Ethical approval for the study was granted by the Institutional
154 Review Board of the Faculty of Dentistry, Universitas Indonesia (approval number:
155 100,701,020). The inclusion criteria specified participants aged 18 years or older who
156 had been undergoing orthodontic treatment with fixed appliances for a minimum

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157 duration of one year and had not consumed probiotics or antibiotics in the preceding 158 three months. Exclusion criteria encompassed individuals with systemic conditions 159 such as hypertension or diabetes, those on systemic medications including 160 antihypertensives, analgesics, hormonal therapies, sedatives, or anti-seizure 161 medications, as well as individuals presenting with severe periodontal disease or 162 known allergies to probiotics.

163 The study utilized *L. reuteri* Prodentis lozenges, obtained from BioGaia 164 (Stockholm, Sweden). Each lozenge (800 mg) contained a minimum of 2×10^8 live 165 *Limosilactobacillus reuteri* Prodentis (previously classified as *Lactobacillus reuteri*). 166 This food supplement, specifically formulated for oral health, included a proprietary 167 combination of the *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289 strains, which 168 are recognized for their potential to support and maintain oral health.

169 To standardize oral hygiene practices, all participants were provided with a 170 standardized toothbrush and toothpaste for use throughout the study period. 171 Participants received oral hygiene instructions and were instructed to brush their teeth 172 twice daily. Each participant was administered one probiotic lozenge containing 173 *Limosilactobacillus reuteri* daily for 14 days, to be taken once per day after morning 174 toothbrushing and prior to breakfast. Plaque samples were collected from participants 175 on the day of enrolment, prior to the initiation of probiotic lozenge consumption, and 176 again on the 14th day following the completion of the probiotic regimen.

177 Before sample collection, mandatory rapid antigen test for SARS-CoV-2 178 detection had to be taken by the subjects in response to the COVID-19 pandemic (as 179 of 30 September 2020 when collecting the samples) and the test result had to be 180 negative for the subject partaking in the study. In brief, the participants were instructed 181 not to eat or drink anything two hours prior to collection of sample. Samples were 182 collected using sterile cotton buds and swabbed from 183 buccal/mesial/distal/lingual/occlusal surfaces of the index teeth of the subjects. The 184 plaque samples were stored in sterile falcon tubes with 5 mL of phosphate-buffered 185 saline (PBS).

186 Furthermore, clinical data, including the Oral Hygiene Index-Simplified (OHI-S) 187 score and the Papilla Bleeding Index (PBI), were recorded at each visit. Previous 188 studies have demonstrated that a 14-day consumption of *Limosilactobacillus reuteri* 189 *Prodentis* significantly reduces clinical parameters such as the Periodontal Index (PI), 190 OHI-S, and Gingival Index (GI) in healthy individuals compared to a placebo

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191 control.[37,38] Overall, twenty subjects had enrolled and fulfilled all requirements and
192 inclusion criteria. Plaque samples from the subjects were continued unto the following
193 downstream analysis.
194

195 **RNA Extraction, cDNA Synthesis and Quantification**

196 RNA from the sample was extracted using TRIzol reagent methodology as
197 instructed by the manufacturer (Thermo Fisher, Waltham, MA). The extracted RNA
198 was synthesized into cDNA using ReverTra Ace™ qPCR RT Master Mix with gDNA
199 Remover (Toyobo, Japan). The mixture for cDNA synthesis is described in Table 1.
200 The cDNA was then quantified using an Invitrogen™ Qubit 3.0 Fluorometer (Thermo
201 Fisher Scientific, Waltham, MA). The cDNA was stored at -20°C for storage of directly
202 used for downstream analysis.
203

204 **qPCR Analysis**

205 Amplification and detection by qPCR (Applied Biosystems, Waltham, MA) were
206 performed. The components of the qPCR Master Mix are listed in Table 2. Using a
207 specific kit, namely HOT FIREpol EvaGreen® qPCR Mix (Solis Biodyne, Tartu,
208 Estonia) which was activated by incubation at 95°C for 10 minutes. This was followed
209 by 40 cycles of denaturation at 95°C for 10 seconds, annealing temperature at
210 60−65°C (Table 3), and elongation at 72°C for 20 seconds. For templates longer than
211 150 bp, the annealing and elongation times were extended to 30 seconds. Actin gene
212 encoding as the housekeeping gene was used for normalization purposes. qPCR was
213 performed on cDNA. qPCR was performed using the primers listed in Table 3.
214

215 **Data Analysis and Outcome Analysis**

216 The data were analysed using the Shapiro−Wilk normality test ($p > 0.05$). For
217 data with a normal distribution, a paired *t*-test was applied. ($p < 0.05$). The software
218 used for the analysis is Statistical Package for the Social Sciences (SPSS) version 27
219 (IBM, Armonk, NY). The outcome analysis are the quantitative data presented from
220 the RT-qPCR instrument. This data can then be correlated with previous study that
221 also conducted such research to ensure the validity of this data.
222
223

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224 **Results:**

225 Based on the results of the qPCR test, the expression of *BCR1* (Graph 1A and
226 1B), *ACE2* (Graph 2A and 2B), *fadA* (Graph 3A and 3B), *aid1* (Graph 4A and 4B), *gelE*
227 (Graph 5A and 5B), and *atlA* (Graph 6A and 6B), on average, were decreased when
228 comparing the day-0 prior to probiotic consumption and day-14 after the subjects
229 consumed the probiotic *L. reuteri*.

230 As shown by the results of the Shapiro−Wilk normality test, all the data were
231 normally distributed ($p > 0.05$). The data were analysed using a paired *t*-test, with a
232 significance level of $p < 0.05$. The results of the paired *t*-test revealed a significant
233 difference in the comparison of the *BCR1* and *ACE2* gene expression data. As shown
234 in Table 4, there was a significant difference ($p < 0.05$) in the expression of *BCR1,*
235 *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes after consuming the probiotic *L. reuteri*.

236

237 **Discussion**:

238 The use of fixed orthodontic appliances often causes poor oral hygiene, thus
239 facilitating microorganism accumulation and various pathological conditions in the oral
240 cavity, such as fungal infections.[39] A previous study revealed an increase in *Candida*
241 in saliva after using fixed orthodontic appliances. The authors attributed this to the
242 design of fixed orthodontic appliances, which creates a space for the retention of food
243 waste.[40] Thus, patients must be instructed about good oral hygiene practices after
244 orthodontic treatment. [38] Fixed orthodontic appliances also induce changes in buffer
245 capacity, salivary flow rates, and acidity (pH), leading to plaque accumulation and an
246 increase in caries and periodontal disease.[41-43] Based on this information, we
247 attempted this study to prove the benefits of consuming probiotics lozenges as a
248 supplement for patients using fixed orthodontic appliances.

249 Biofilm formation is an important virulence factor of *F. nucleatum* due to its higher
250 resistance to host defence or antibacterial agents compared to planktonic cells.[44]
251 Several studies detected increased numbers of *Porphyromonas gingivalis, F.*
252 *nucleatum, P. intermedia,* and *Tannerella forsythia* after the use of fixed orthodontic
253 appliances.[15,45,46] They also reported that *F. nucleatum* increased the risk of
254 periodontitis in orthodontic patients due to a conducive environment for anaerobic
255 bacteria.[15,45,46] Biofilm formation is also a contributing factor to *E. faecalis* colonization
256 and infection. Biofilms develop through various processes by which bacteria adhere

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257 to surfaces, decompose complex matrices, and develop into bacterial colonies, which
258 adhere to surfaces.[47]

259 In this study, we used the qPCR method and $2^{-\Delta\Delta CT}$ formula to calculate target
260 gene expression. As shown by the results, the probiotic *L. reuteri* affected the
261 expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes and the formation of *C.*
262 *albicans, F. nucleatum*, and *E. faecalis* biofilms. Based on the Shapiro−Wilk normality
263 test, all the data were normally distributed, with $p > 0.05$. The paired *t*-test results
264 revealed significant differences in the expression *of BCR1, ACE2, fadA*, *aid1*, *gelE*,
265 and *atlA* genes (all $p < 0.05$).

266 Based on the results of this study, the probiotic *L. reuteri* significantly
267 downregulated the transcription of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes. In
268 gene expression, the process of translating genetic information in the form of
269 sequence of bases of DNA or RNA into proteins.[48] Through gene expression
270 measurement, it is possible to assess qualitatively and quantitatively the effect of a
271 treatment, such as the administration of a drug compound.[49] Gene expression in
272 microorganisms is involved in regulating cell-cell communication, carbohydrate
273 metabolism, adherence, and adaptation to the surrounding environment. A decrease
274 in the expression of specific genes can reduce microorganism colonization and
275 microorganism numbers.[50]

276 *BCR1*, a major gene transcription factor, produces an adhesin protein, which
277 facilitates *C. albicans* attachment to mucosal surfaces, which is a critical stage of
278 infection.[51] Deletion of *BCR1* eliminates *C. albicans* gene function, resulting in a
279 decrease in biofilm formation.[52] This was supported by the results of the present study,
280 which revealed a statistically significant decrease in *BCR1* gene expression after
281 consuming the probiotic.

282 The expression of *ACE2* also decreased based on the results of the statistical
283 tests. With deletion of *ACE2*, *C. albicans* is unable to form hyphal cells, and thus
284 biofilm formation is inhibited.[30] An earlier in vitro study showed that probiotics have
285 antifungal effects against *C. albicans* in the oral cavity. Regular use of probiotics
286 helped to inhibit *Candida* biofilms and reduced *Candida* colonization in the oral cavity,
287 thereby reducing the possibility of candidiasis infection.[53]

288 FadA protein is the main *F. nucleatum* virulence factor and mediates microbial
289 attachment and colonization.[8] Based on the results of this study, the probiotic *L. reuteri*

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290 appears to influence the pathogenicity of *F. nucleatum* adhesion molecules and
291 colonization and affect biofilm formation through decreased expression of the *fadA*
292 gene.[35] Various *F. nucleatum* adhesins mediate adhesion and aggregation and
293 function as coaggregation intermediaries in the formation and maturation of dental
294 biofilms.[54] The interaction of Fusobacterium with other species is largely mediated by
295 the adhesin genes *radD* and *aid1*.[55] The *aid1* gene plays a role in interspecies
296 interactions, colonization, and aggregation of *F. nucleatum*. In a previous study,
297 inactivation of the *aid1* gene decreased the ability of *F. nucleatum* to aggregate,
298 especially with *Streptococcus* spp. or *E. faecalis*.[14] As shown in earlier studies,
299 probiotics can affect the expression of genes involved in cell adhesion, quorum
300 sensing (QS), virulence factors, and biofilm formation.[56,57]

301 The *E. faecalis gelE* gene has the ability to hydrolyse gelatine, collagen, fibrin,
302 and other peptides.[58] Gene *gelE* is a virulence factor in infection formation through
303 bacterial attachment and biofilm formation.[22] In *E. faecalis*, biofilm formation is
304 regulated by QS, where Fsr regulates the expression of the *gelE* gene.[59] Fsr regulates
305 the formation of *E. faecalis* biofilms through its product *gelE* and serine proteases.[60]
306 QS is a molecular mechanism by which bacterial cells communicate with each other
307 via signalling molecules in biofilms. If the protease encoded by a signalling factor is
308 decreased, communication between bacteria and biofilm formation will be disrupted.[57]
309 GelE can also activate *atlA*, which is responsible for eDNA release at the biofilm
310 maturation stage.[61]

311 AtlA is involved in the hydrolysis of peptidoglycan, which plays an important role
312 in separating cells division after replication.[22] AtlA plays a role in the biofilm maturation
313 stage of *E. faecalis*, during which eDNA is released and contributes to biofilm
314 attachment and stability, biofilm defects in primary attachment, and decreased biofilm
315 production.[20,23] This study focused on the probiotic *L. reuteri*, which has ability to
316 secrete antimicrobial substances and compete with oral pathogens for adhesion to
317 mucosa. In addition, *L. reuteri* can adapt and change the pH of the surrounding
318 environment, thereby inhibiting the growth of oral pathogens.[62] The antimicrobial
319 substances secreted by *L. reuteri* are reuterin and reutericycline.[63] Reuterin and
320 reutericycline are broad-spectrum antimicrobial agents that are effective against gram-
321 positive and negative bacteria, fungi, and protozoa by inhibiting microbial DNA
322 synthesis.[35,41]

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323 Many dental and oral health care products used daily now include probiotics. The
324 use of probiotics is increasing due to their advantages over chemical agents, namely
325 reducing the risk of antibiotic resistance.[64] Probiotics work by modulating the immune
326 system, producing antimicrobial substances, and inhibiting certain pathogenic
327 organisms by interfering with adhesion, colonization, and biofilm formation. They
328 inhibit the growth of pathogens via the production of various substances, such as lactic
329 acid and acetic acid, which penetrate the bacterial cell membrane and lower the
330 cytoplasmic pH of pathogenic bacteria. Hydrogen peroxide and bacteriocin can
331 destroy the cell membrane of pathogenic bacteria and inhibit the synthesis of
332 pathogenic DNA.[32,33,35,65] Based on the results of this study, 2 weeks of daily
333 consumption of the probiotic *L. reuteri* affected the process of biofilm formation by
334 downregulating the expression of *BCR1*, *ACE2, fadA, aid1, gelE,* and *atlA g*enes,
335 which function as adherent regulators and regulators of hyphae formation in biofilm
336 formation. Many previous studies demonstrated that the addition of probiotics to dental
337 and oral health care products. Probiotic can reduced pathogenic microorganisms in
338 plaque samples from patients using fixed orthodontic appliances.[41,64,66–68] Therefore,
339 it can be stated that the probiotic *L. reuteri* has good ability as an additional treatment
340 for dental and oral health in patients using fixed orthodontic appliances.
341 The limitation of this study is the small sample size and the fact that the sampling
342 had to be done during COVID-19 pandemic. Although the sample size is small, the
343 result of this study can still provide concise and significant result. In the future, there
344 should be larger sample size for conducting this research so the result can more
345 accurately represents the actual population. Daily consumption of probiotic lozenges
346 duration can also be increased for more precise and accurate results. On the other
347 hand, consumption of probiotic lozenges research can also be conducted for other
348 aspect of oral health diseases to promote the functionality and health-inducing aspect
349 of probiotic lozenges, such as antiinflammation.
350

351 **Conclusion:**

352 Within the limitation of this study, it can be concluded that the probiotic *L. reuteri*
353 influences the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in biofilm
354 formation. By reducing the expression of those genes, the probiotic *L. reuteri* can

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355 reduce biofilm formation such as dental plaque in patients using fixed orthodontic
356 appliances.
357
358 **Clinical significance:** Consumption of probiotic lozenges were confirmed to reduce
359 bacterial and fungal biofilm, as proven by the reduction of virulence gene expression,
360 hence, helping increasing oral health of consumer. The results of this study help
361 clinicians provide probiotic lozenges for patients to promote and maintain their oral
362 health.
363
364

365 **List of abbreviations:**

366 ^{o}C : Celcius degree
367 ACE2 : Angiotensin Converting Enzyme 2
368 aid1 : adherence inducing determinant gene 1
369 atlA : autolysin
370 BCR1 : Biofilm and Cell wall Regulator 1
371 cDNA : copy Deoxyribonucleic Acid
372 CFU/mL : Colony forming unit per mililiter
373 DNA : Deoxyribonucleic Acid
374 eDNA : extracellular Deoxyribonucleic Acid
375 fadA : fusobacterium adhesion A
376 gelE : gelatinase
377 radD : arginine-inhibitable adhesin
378 RT-qPCR : Quantitative Polymerase Chain Reaction
379

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380 **References:**

381 1. Sihotang BM, Panjaitan M, Sitanggang JM, Putri A, Harahap FR. Perbedaan oral
382 hygiene pemakaian pesawat ortodonti cekat dan pesawat ortodonti lepasan
383 pada pasien klinik spesialis RS Herna. Jurnal Prima Medika Sains. 2020 Mar
384 15;2(1):1–5.
385 2. Gkantidis N, Christou P, Topouzelis N. The orthodontic-periodontic
386 interrelationship in integrated treatment challenges: a systematic review. J Oral
387 Rehabil. 2010 May 1;37(5):377–90.
388 3. Oktaviani V, Santoso O. Perbedaan Indeks Higiene Oral dan pH Plak Kelompok
389 Pemakai dan Bukan Pemakai Pesawat Ortodonti Cekat. 2016;5(1):8.
390 4. Bowen WH, Burne RA, Wu H, Koo H. Oral Biofilms: Pathogens, Matrix and
391 Polymicrobial Interactions in Microenvironments. Trends Microbiol. 2018
392 Mar;26(3):229–42.
393 5. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence.
394 2011;2(5):435–44.
395 6. Verrusio C, Iorio-Siciliano V, Blasi A, Leuci S, Adamo D, Nicolò M. The effect of
396 orthodontic treatment on periodontal tissue inflammation: A systematic review.
397 Quintessence Int. 2018;49(1):69–77.
398 7. Perkowski K, Baltaza W, Conn DB, Marczyńska-Stolarek M, Chomicz L.
399 Examination of oral biofilm microbiota in patients using fixed orthodontic
400 appliances in order to prevent risk factors for health complications. Ann Agric
401 Environ Med. 2019 Jun 17;26(2):231–5.
402 8. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. Curr Opin
403 Microbiol. 2015 Feb;0:141–7.
404 9. Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS. Bacterial
405 coaggregation: an integral process in the development of multi-species biofilms.
406 Trends Microbiol. 2003 Feb;11(2):94–100.
407 10. Thurnheer T, Karygianni L, Flury M, Belibasakis GN. Fusobacterium Species and
408 Subspecies Differentially Affect the Composition and Architecture of Supra- and
409 Subgingival Biofilms Models. Front Microbiol. 2019 Jul 30;10:1716.
410 11. Chew J, Zilm PS, Fuss JM, Gully NJ. A proteomic investigation of Fusobacterium
411 nucleatum alkaline-induced biofilms. BMC Microbiology. 2012 Sep 3;12(1):189.
412 12. Kaplan CW, Lux R, Haake SK, Shi W. The Fusobacterium nucleatum Outer
413 Membrane Protein RadD Is an Arginine-Inhibitable Adhesin Required for Inter-
414 Species Adherence and the Structured Architecture of Multi-Species Biofilm. Mol
415 Microbiol. 2009 Jan;71(1):35–47.
416 13. Cochrane K, Robinson AV, Holt RA, Allen-Vercoe E. A survey of Fusobacterium
417 nucleatum genes modulated by host cell infection. Microb Genom. 2020
418 Feb;6(2).
419 14. Kaplan A, Kaplan CW, He X, McHardy I, Shi W, Lux R. Characterization of aid1,
420 a novel gene involved in Fusobacterium nucleatum interspecies interactions.
421 Microb Ecol. 2014 Aug;68(2):379–87.
422 15. Liu P, Liu Y, Wang J, Guo Y, Zhang Y, Xiao S. Detection of Fusobacterium
423 Nucleatum and fadA Adhesin Gene in Patients with Orthodontic Gingivitis and
424 Non-Orthodontic Periodontal Inflammation. PLOS ONE. 2014 Jan
425 9;9(1):e85280.

426 16. Brennan CA, Garrett WS. Fusobacterium nucleatum - symbiont, opportunist and
427 oncobacterium. Nat Rev Microbiol. 2019 Mar;17(3):156–66.
428 17. Wu T, Cen L, Kaplan C, Zhou X, Lux R, Shi W, et al. Cellular Components
429 Mediating Coadherence of Candida albicans and Fusobacterium nucleatum. J
430 Dent Res. 2015 Oct;94(10):1432–8.
431 18. Wang Q-Q, Zhang C-F, Chu C-H, Zhu X-F. Prevalence of Enterococcus faecalis
432 in saliva and filled root canals of teeth associated with apical periodontitis. Int J
433 Oral Sci. 2012 Apr;4(1):19–23.
434 19. Najafi K, Gholizadeh P, Tanomand A, Ahangarzadeh Rezaee M, Mahmood S,
435 Asgharzadeh M, et al. Oral Cavity infection by Enterococcus Faecalis: virulence
436 factors and pathogenesis. Reviews in Medical Microbiology. 2019 Jan 2;30.
437 20. Ch'ng J-H, Chong KKL, Lam LN, Wong JJ, Kline KA. Biofilm-associated infection
438 by enterococci. Nat Rev Microbiol. 2019 Jan;17(2):82–94.
439 21. Anderson AC, Jonas D, Huber I, Karygianni L, Wölber J, Hellwig E, et al.
440 Enterococcus faecalis from Food, Clinical Specimens, and Oral Sites:
441 Prevalence of Virulence Factors in Association with Biofilm Formation. Front
442 Microbiol. 2016 Jan 11;6:1534.
443 22. Stinemetz EK, Gao P, Pinkston KL, Montealegre MC, Murray BE, Harvey BR.
444 Processing of the major autolysin of E. faecalis, AtlA, by the zinc-
445 metalloprotease, GelE, impacts AtlA septal localization and cell separation.
446 PLOS ONE. 2017 Oct 19;12(10):e0186706.
447 23. Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, et al. Contribution of
448 Autolysin and Sortase A during Enterococcus faecalis DNA-Dependent Biofilm
449 Development. Infect Immun. 2009 Sep;77(9):3626–38.
450 24. Paganelli FL, Willems RJL, Jansen P, Hendrickx A, Zhang X, Bonten MJM, et al.
451 Enterococcus faecium Biofilm Formation: Identification of Major Autolysin
452 AtlAEfm, Associated Acm Surface Localization, and AtlAEfm-Independent
453 Extracellular DNA Release. mBio. 2013 Apr 16;4(2):e00154-13.
454 25. Putri MH, Herijulianti E. Ilmu Pencegahan Penyakit Jaringan Keras dan Jaringan
455 Pendukung Gigi | OPAC Integrasi | Online Public Access Catalog | Universitas
456 Gadjah Mada. RGC; 2010.
457 26. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent
458 public health problem. Clin Microbiol Rev. 2007;20(1):133–63.
459 27. Garcia MC, Lee JT, Ramsook CB, Alsteens D, Dufrêne YF, Lipke PN. A Role for
460 Amyloid in Cell Aggregation and Biofilm Formation. PLOS ONE.
461 2011;6(3):e17632.
462 28. Nobile CJ, Mitchell AP. Regulation of Cell-Surface Genes and Biofilm Formation
463 by the C. albicans Transcription Factor Bcr1p. Current Biology. 2005 Jun
464 21;15(12):1150–5.
465 29. Mulhern SM, Logue ME, Butler G. Candida albicans Transcription Factor Ace2
466 Regulates Metabolism and Is Required for Filamentation in Hypoxic Conditions.
467 Eukaryot Cell. 2006 Dec;5(12):2001–13.
468 30. Saputo S, Kumar A, Krysan DJ. Efg1 Directly Regulates ACE2 Expression To
469 Mediate Cross Talk between the cAMP/PKA and RAM Pathways during Candida
470 albicans Morphogenesis. Eukaryot Cell. 2014 Sep;13(9):1169–80.

471 31. Widyarman AS, Bachtiar EW, Bachtiar BM, Seneviratne CJ. Inhibitory effect of
472 probiotic lactobacilli against Streptococcus mutans and Porphyromonas
473 gingivalis biofilms. Scientific Dental Journal. 2019 May 1;3(2):50.
474 32. Widyarman AS, Ramajayanti T, Theodorea CF. Indonesian Strain of Probiotic
475 Reduces the Initial Biofilm Colonization. The Open Dentistry Journal [Internet].
476 2020 Nov 13 [cited 2022 Feb 13];14(1). Available from:
477 https://opendentistryjournal.com/VOLUME/14/PAGE/544/FULLTEXT/
478 33. Widyarman AS, Theodorea CF. Novel Indigenous Probiotic Lactobacillus reuteri
479 Strain Produces Anti-biofilm Reuterin against Pathogenic Periodontal Bacteria.
480 Eur J Dent [Internet]. 2021 Jul 24 [cited 2022 Feb 13]; Available from:
481 http://www.thieme-connect.de/DOI/DOI?10.1055/s-0041-1731591
482 34. Mu Q, Tavella VJ, Luo XM. Role of Lactobacillus reuteri in Human Health and
483 Diseases. Frontiers in Microbiology [Internet]. 2018 [cited 2022 Feb 13];9.
484 Available from: https://www.frontiersin.org/article/10.3389/fmicb.2018.00757
485 35. Department of Microbiology, Faculty of Dentistry, Trisakti University, Jakarta,
486 11440 Indonesia, Sari Widyarman A, Kiky Elysia Lazaroni N, Undergraduate
487 Program, Faculty of Dentistry, Trisakti University, Jakarta, 11440 Indonesia.
488 Persistent Endodontics Pathogens Biofilm Inhibited by Lactobacillus reuteri
489 Indonesian Strain Lactobacillus reuteri Indonesian. J Dent Indones [Internet].
490 2019 Dec 31 [cited 2022 Feb 13];26(3). Available from:
491 https://scholarhub.ui.ac.id/jdi/vol26/iss3/7/
492 36. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of
493 probiotic and pathogen strains. Eur Food Res Technol. 2008;226(5):1065–73.
494 37. Twetman S, Derawi B, Keller M, et al. Short-term effect of chewing gums
495 containing probiotic Lactobacillus reuteri on the levels of inflammatory mediators
496 in gingival crevicular fluid. Acta Odontol Scand. 2009;67(1):19–24.
497 38. Krasse P, Carlsson B, Dahl C, et al. Decreased gum bleeding and reduced
498 gingivitis by the probiotic Lactobacillus reuteri. Swed Dent J. 2006;30(2):55–60.
499
500 39. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. J Oral
501 Microbiol [Internet]. 2011;3. Available from:
502 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3087208/
503 40. Khanpayeh E, Jafari AA, Tabatabaei Z. Comparison of salivary Candida profile
504 in patients with fixed and removable orthodontic appliances therapy. Iran J
505 Microbiol. 2014 Aug;6(4):263–8.
506 41. Widyarman AS, Hartono V, Marjani LI, Irawan D, Luthfi L, Bachtiar BM.
507 Lactobacillus reuteri containing Probiotic Lozenges Consumption Reduces
508 Streptococcus mutans, Streptococcus sobrinus, Porphyromonas gingivalis, and
509 Aggregatibacter actinomycetemcomitans in Orthodontic Patients. Journal of
510 International Dental and Medical Research. 2018 Jan 1;11(2):628–33.
511 42. Restrepo M, Bussaneli DG, Jeremias F, Cordeiro RCL, Raveli DB, Magalhães
512 AC, et al. Control of White Spot Lesions with Use of Fluoride Varnish or
513 Chlorhexidine Gel During Orthodontic Treatment A Randomized Clinical Trial. J
514 Clin Pediatr Dent. 2016;40(4):274–80.

JAYPEE

515 43. Lu H, Tang H, Zhou T, Kang N. Assessment of the periodontal health status in
516 patients undergoing orthodontic treatment with fixed appliances and Invisalign
517 system. Medicine (Baltimore). 2018 Mar 30;97(13):e0248.
518 44. Fitriasari N, Wahyuni IS. POTENSI PROBIOTIK DALAM TATALAKSANA ORAL
519 CANDIDIASIS: Ulasan Sistematik. ODONTO : Dental Journal. 2021 Jul
520 12;8(1):34–44.
521 45. Guo L, Feng Y, Guo H-G, Liu B-W, Zhang Y. Consequences of orthodontic
522 treatment in malocclusion patients: clinical and microbial effects in adults and
523 children. BMC Oral Health. 2016 Oct 28;16:112.
524 46. Alqerban A. Efficacy of antimicrobial photodynamic and photobiomodulation
525 therapy against Treponema denticola, fusobacterium nucleatum and human beta
526 defensin-2 levels in patients with gingivitis undergoing fixed orthodontic
527 treatment: A clinic-laboratory study. Photodiagnosis and Photodynamic Therapy.
528 2020 Mar 1;29:101659.
529 47. Willett JLE, Ji MM, Dunny GM. Exploiting biofilm phenotypes for functional
530 characterization of hypothetical genes in Enterococcus faecalis. npj Biofilms
531 Microbiomes. 2019 Sep 19;5(1):1–14.
532 48. Sinaga E. Regulasi Ekspresi Gen. Regulasi Ekspresi Gen. 2011;1–24.
533 49. Van Peer G, Mestdagh P, Vandesompele J. Accurate RT-qPCR gene expression
534 analysis on cell culture lysates. Sci Rep. 2012 Jan 13;2:222.
535 50. Shemesh M, Tam A, Steinberg D 2007. Expression of biofilm-associated genes
536 of Streptococcus mutans in response to glucose and sucrose. Journal of Medical
537 Microbiology. 56(11):1528–35.
538 51. Nikoomanesh F, Department of medical mycology, Faculty of medical science,
539 Tarbiat Modares University, Tehran, Iran, Roudbarmohammadi S, Department
540 of medical mycology, Faculty of medical science, Tarbiat Modares University,
541 Tehran, Iran, Roudbary M, Department of Medical Mycology and Parasitology,
542 School of Medicine, Iran University of Medical Sciences, Tehran, Iran, et al.
543 Investigation of bcr1 Gene Expression in Candida albicans Isolates by RT-PCR
544 Technique and its Impact on Biofilm Formation. iem. 2016 Jan 1;2(1):22–4.
545 52. Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP. Divergent Targets
546 of Candida albicans Biofilm Regulator Bcr1 In Vitro and In Vivo. Eukaryotic Cell.
547 2012 Jul;11(7):896–904.
548 53. Matsubara VH, Bandara HMHN, Mayer MPA, Samaranayake LP. Probiotics as
549 Antifungals in Mucosal Candidiasis. Clin Infect Dis. 2016;62(9):1143–53.
550 54. Li Q, Tan L, Wang H, Kou Y, Shi X, Zhang S, et al. Fusobacterium nucleatum
551 Interaction with Pseudomonas aeruginosa Induces Biofilm-Associated Antibiotic
552 Tolerance via Fusobacterium Adhesin A. ACS Infect Dis. 2020 Jul 10;6(7):1686–
553 96.
554 55. Kaplan A. Identification and Characterization of a Novel Lipoprotein in
555 Fusobacterium nucleatum [Internet]. UCLA; 2015 [cited 2022 Feb 13]. Available
556 from: https://escholarship.org/uc/item/3r90n33c
557 56. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-
558 time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001
559 Dec;25(4):402–8.

JAYPEE

560 57. Barzegari A, Kheyrolahzadeh K, Hosseiniyan Khatibi SM, Sharifi S, Memar MY,
561 Zununi Vahed S. The Battle of Probiotics and Their Derivatives Against Biofilms.
562 Infect Drug Resist. 2020 Feb 26;13:659–72.
563 58. Guneser MB, Eldeniz AU. The effect of gelatinase production of Enterococcus
564 faecalis on adhesion to dentin after irrigation with various endodontic irrigants.
565 Acta Biomater Odontol Scand. 2016 Nov 15;2(1):144–9.
566 59. Hashem YA, Amin HM, Essam TM, Yassin AS, Aziz RK. Biofilm formation in
567 enterococci: genotype-phenotype correlations and inhibition by vancomycin. Sci
568 Rep. 2017 Jul 18;7(1):5733.
569 60. Wang L, Dong M, Zheng J, Song Q, Yin W, Li J, et al. Relationship of biofilm
570 formation and gelE gene expression in Enterococcus faecalis recovered from
571 root canals in patients requiring endodontic retreatment. J Endod. 2011
572 May;37(5):631–6.
573 61. Ali L, Goraya MU, Arafat Y, Ajmal M, Chen J-L, Yu D. Molecular Mechanism of
574 Quorum-Sensing in Enterococcus faecalis: Its Role in Virulence and Therapeutic
575 Approaches. Int J Mol Sci. 2017 May 3;18(5):960.
576 62. Bonifait L. Probiotics for Oral Health: Myth or Reality? 2009;75(8):6.
577 63. Urbańska M, Szajewska H. The efficacy of Lactobacillus reuteri DSM 17938 in
578 infants and children: a review of the current evidence. Eur J Pediatr.
579 2014;173(10):1327–37.
580 64. Benic GZ, Farella M, Morgan XC, Viswam J, Heng NC, Cannon RD, et al. Oral
581 probiotics reduce halitosis in patients wearing orthodontic braces: a randomized,
582 triple-blind, placebo-controlled trial. J Breath Res. 2019 May 31;13(3):036010.
583 65. Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A.
584 Probiotic mechanisms of action. Ann Nutr Metab. 2012;61(2):160–74.
585 66. Widyarman AS, Yunita ST, Prasetyadi T. Consumption of Yogurt Containing
586 Probiotic *Bifidobacterium lactis* Reduces *Streptococcus mutans* in Orthodontic
587 Patients. Scientific Dental Journal. 2018 Jan 25;2(1):19–25.
588 67. Alp S, Baka ZM. Effects of probiotics on salivary *Streptecoccus mutans* and
589 Lactobacillus levels in orthodontic patients. Am J Orthod Dentofacial Orthop.
590 2018 Oct;154(4):517–23.
591 68. Goyal N, Shamanna PU, Varughese ST, Abraham R, Antony B, Emmatty R, et
592 al. Effects of amine fluoride and probiotic mouthwash on levels of
593 *Porphyromonas gingivalis* in orthodontic patients: A randomized controlled trial.
594 J Indian Soc Periodontol. 2019;23(4):339–44.
595 69. Ranjith K, Kalyana Chakravarthy S, Adicherla H, Sharma S, Shivaji S. Temporal
596 Expression of Genes in Biofilm-Forming Ocular *Candida albicans* Isolated From
597 Patients With Keratitis and Orbital Cellulitis. Investigative Ophthalmology &
598 Visual Science. 2018 Jan 25;59(1):528–38.
599 70. Nailis H, Coenye T, Van Nieuwerburgh F, Deforce D, Nelis HJ. Development and
600 evaluation of different normalization strategies for gene expression studies in
601 *Candida albicans* biofilms by real-time PCR. BMC Mol Biol. 2006 Aug 4;7:25.
602 71. Vos M, Quince C, Pijl AS, Hollander M de, Kowalchuk GA. A Comparison of rpoB
603 and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity.
604 PLOS ONE. 2012 Feb 15;7(2):e30600.

JAYPEE

605 72. Zheng J-X, Wu Y, Lin Z-W, Pu Z-Y, Yao W-M, Chen Z, et al. Characteristics of
606 and Virulence Factors Associated with Biofilm Formation in Clinical
607 Enterococcus faecalis Isolates in China. Front Microbiol. 2017 Nov 24;8:2338

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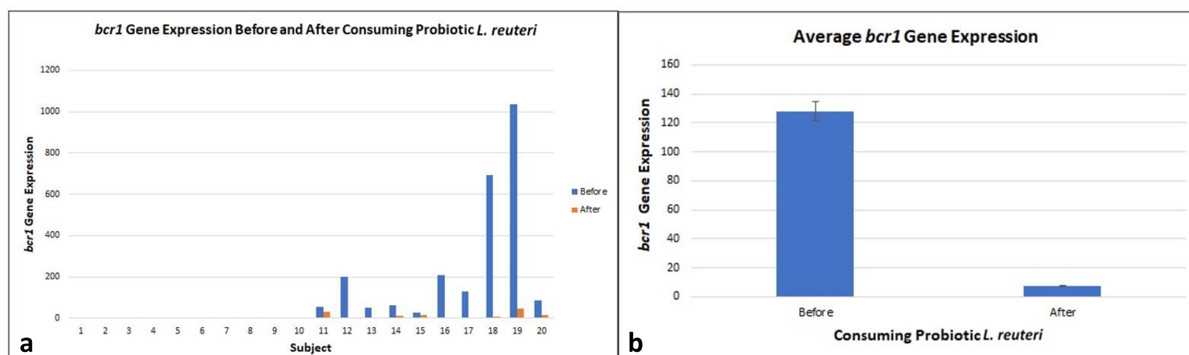
611 ## Figures and figure legends
612



613
614 **Graph 1**. (**a**) A graph showing bcr1 gene expression in plaque samples *(N*= 20) as
615 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
616 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
617 *bcr1* gene expression in plaque samples as assessed by the RT-qPCR method and
618 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
619 lozenges.
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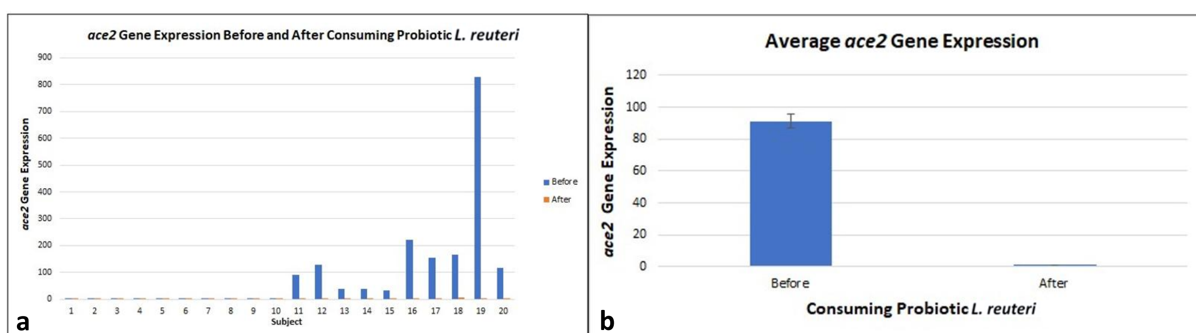

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622
623 **Graph 2**. (**a**) A graph showing ace2 gene expression in plaque samples *(N*= 20) as
624 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
625 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
626 *ace2* gene expression in plaque samples as assessed by the RT-qPCR method and
627 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
628 lozenges.
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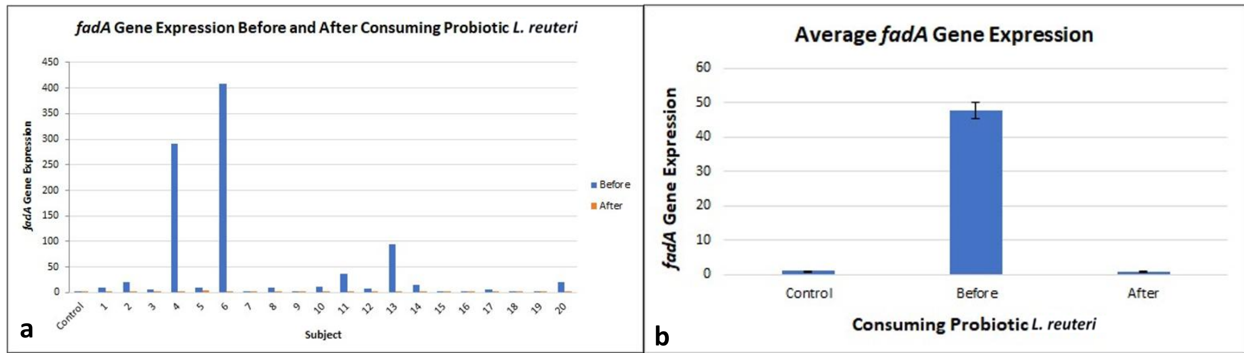

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636 **Graph 3**. (**a**) A graph showing fadA gene expression in plaque samples *(N*= 20) as
637 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
638 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
639 *fadA* gene expression in plaque samples as assessed by the RT-qPCR method and
640 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
641 lozenges.
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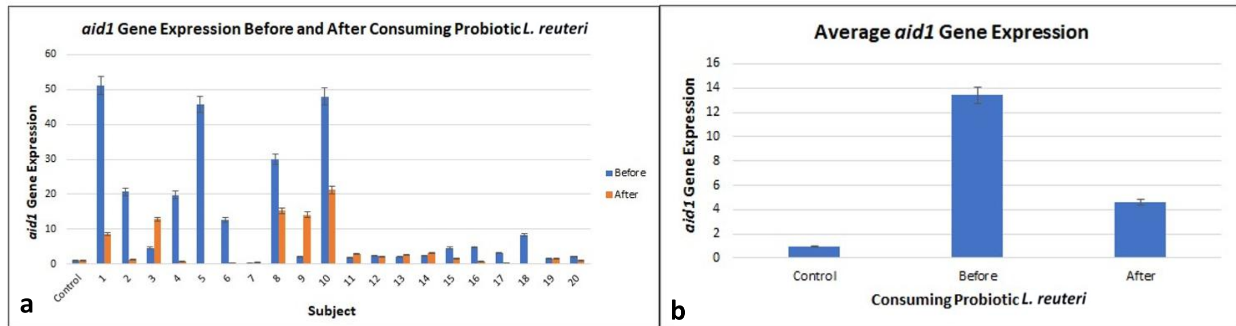

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646 **Graph 4**. (**a**) A graph showing aid1 gene expression in plaque samples *(N*= 20) as
647 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
648 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
649 *aid1* gene expression in plaque samples as assessed by the RT-qPCR method and
650 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
651 lozenges.
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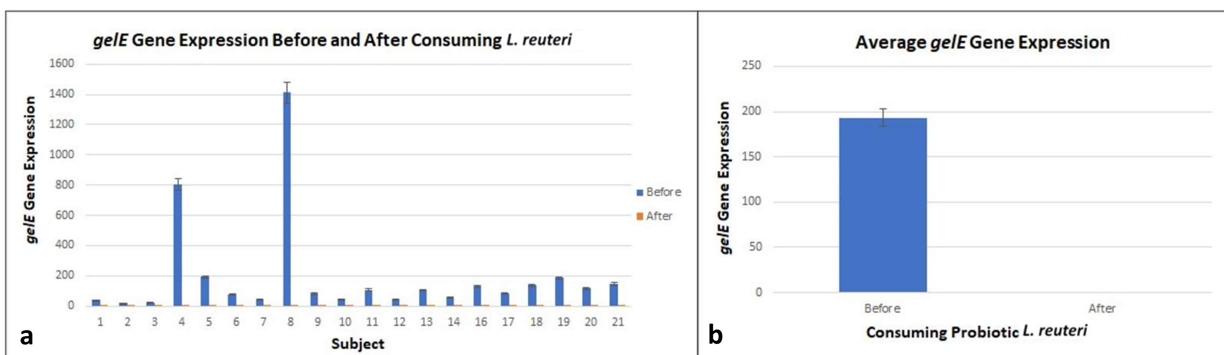
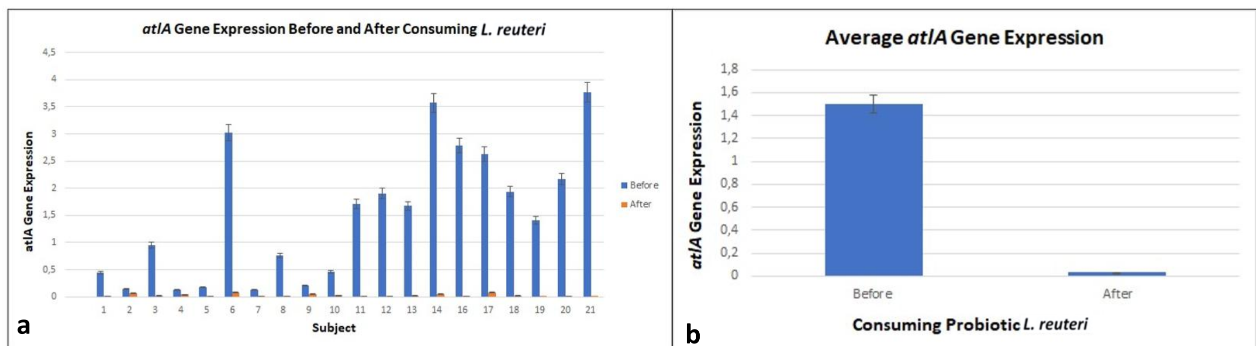


653
654 **Graph 5.** (**a**) A graph showing gelE gene expression in plaque samples *(N*= 20) as
655 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
656 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of

gelE gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges.



Graph 6. (**a**) A graph showing atlA gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of *atlA* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges.

676 ## Tables and table legends

677

678 **Table 1.** Reagent components for DNase I Reaction Solution

Component	**Volume**
4x DN *Master Mix*	2 mL
RNA *template*	0,5 pg – 0,5 mg
Nuclease-free Water	X mL
Total Volume	**8 mL**

679

680 **Table 2.** Components of Master Mix RT-qPCR

Component	**Volume**
5 x HOT FIREPol EvaGreen® qPCR Mix Plus	4 mL
Primer *Forward*	1 mL
Primer *Reverse*	1 mL
DNA *template*	2 mL
NFW	12 mL
Total	**20 mL**

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684 **Table 3.** *Primer sequence* for RT-qPCR

Genes	*Primer sequence*	
BCR1[51]	*forward*: 5’-CTTCAGCAGCTTCATTAACACCTA-3’ *reverse*: 5’-TCTTGGATCAGGTGTACTTTTCAA-3’	Initial denaturation of 95°C for 5 minutes; 40 Cycles of denaturation at 95°C for 1 minute and annealing at 58°C for 1 minute.
ACE2[69]	*forward* 5’-AGAATTGACCGTTGTCCGTGTAAG-3’ *reverse*: 5’-AATGGGTGAATAAATCCCTCCCTAA-3’	Initial denaturation 95°C for 2 minutes; 40 Cycles of denaturation at 95°C for 30 seconds and annealing at 60°C for 1 minute.
Housekeeping gene C. albicans : *ACT1*[70]	*forward*: 5’-TTTCATCTTCTGTATCAGAGGAACTTATTT-3’ *reverse*: 5’-ATGGGATGAATCATCAAACAAGAG-3’	Initial denaturation 95°C for 10 minutes; 40 Cycles of denaturing 95°C for 15 seconds and annealing 60°C for 1 minute

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fadA[15]	*forward*: 5' -CAC AAG CTG ACG CTG CTA GA- 3' *reverse*: 5' -TTA CCA GCT CTT AAA GCT TG- 3'	Initial incubation for 4 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55.8°C for 30 seconds, and elongation at 72°C for 40 seconds and the final elongation for 6 min.[14]
aid1[14]	*forward:* 5' -TACAGGAG GTGCCGTAGCAG-3' *reverse:* 5' -TTTTTGTTAATTCT CCAGCTCCA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.[13]
Housekeeping gene *F. nucleatum*: *rpoB*[71]	*forward:* 5'-GGYTWYGAAGTNCGHGACGTDCA- 3' *reverse:* 5'-TGACGYTGCATGTTBGMR CCCATMA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.

gelE[60]	*forward:* 5'- CGGAACATACTGCCGGTTTAGA - 3' *reverse:* 5'- TGGATTAGATGCACCCGAAAT - 3'	Initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 5 seconds, and annealing at 60°C for 30 seconds.
atlA[72]	*forward:* 5'- AATAATCAATCAGGAACGAATACG - 3' *reverse:* 5'- GCCACACTAACACCGAAT -3'	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.
Housekeeping gene E. faecalis: rpoA[72]	*forward:* 5'- GTGAAACCTGGTCGTGGCTA - 3' *reverse:* 5'- CGACGAACGGGTGTGTAGAT- 3'	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.

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5. Bukti konfirmasi submit revisi, respon kepada reviewer, dan artikel yang diresubmit

16 Januari 2025

Joko Kusnoto <joko.k@trisakti.ac.id>

JCDP_24_408_R3 has been sent for review

1 message

noreply@jaypeejournals.com <noreply@jaypeejournals.com> Thu, Jan 16, 2025 at 5:00 PM
To: armeliasari@trisakti.ac.id, joko.k@trisakti.ac.id, sarasafirah@gmail.com, litayanas@gmail.com, winnie.valentini@gmail.com
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Dear Dr. Armelia Sari Widyarman,

The article **Lactobacillus reuteri Probiotic Consumption reduced Various Virulence Gene Expression in Dental Plaque of Fixed Orthodontic Subjects** submitted by you through Scriptor's Zone in Journal The Journal of Contemporary Dental Practice has passed the preliminary stage and has now been further sent to Editor/Reviewers for peer-review process.

Once the review is over, you will be informed of the Editorial decision via e-mail.

We thank you for your contribution to the journal and look forward for a continued support.

Thanking you

Managing Editor - Jaypee Journals
The Journal of Contemporary Dental Practice

No.	Reviewer's Comment	Author's Response	Location of Revision
1	Prepare a **short running title** for your manuscript to be used as header for each page. (Length: not more than 10 words)	Thank you for the revision. The short running title has been added onto the header of each page.	Header
2	**Abstract**: In results section please mention p value	We thank you for the instructive comment. We have added it in the abstract.	Page 1 line 18
3	Figures: provide representative gene images, rename all the bar graphs as graph 1, 2 etc and place all tables, graphs, figures at the end of the main article with legends	We are grateful for the comment. We have provided the revision as per instructed.	Page 18-24
4	Explain the need to perform the study, the novelty and then finally, end with a specific aim of the study.	Thank you for your constructive comment. We have added the information regarding the additional information in the paper.	Page 3 line 80 – page 4 line 98
5	Brief about the genes assessed in the study	Thank you for your kind comment. Each gene has been discussed briefly in the introduction.	Page 2 line 42 – page 3 line 79

6	Elaborate inclusion and exclusion criteria. Here mention which type of fixed appliance were included in the study	We thank you for the specific revision comment. The revision has been made in the passage.	Page 4 line 102 – 113
7	Explain if study groups were there ie control or only before and after comparison was done	Thank you for the constructive comments. The information has been described in the paper.	Page 4 line 120 – 127
8	Justify sample size calculation	Thank you for the constructive comment. We used twenty subjects for this study due to this study being a preliminary stage of using probiotic lozenges to confirm its beneficial effects on patients using fixed orthodontic appliances and has been calculated using the formula: $n = \frac{Z^2.N.p.q}{d^2(N-1)+Z^2.p.q}$	
9	Explain randomization and blinding method	We appreciated your kind comment. We categorized this clinical trial as an open-labelled prospective clinical trial. As the study was conducted during the COVID-19 pandemic, shipment of placebos from outside Indonesia was prohibited. In addition, finding subjects willing to participate in this study was also challenging on its own.	Page 4 line 102-103

10	Explain inference of the study.	Thank you for the instructive criticism. We have inferred the result of this study and added it in the paper.	Page 7 line 175 – 185
11	First explain why this study was conducted	We are grateful for the comments. We have relayed the information in the paper.	Page 7 line 188-198
12	Then justify the material used and technique used for outcome analysis with references	Thank you for the constructive comment. We analysed the gene expression level using qPCR instrument with $2^{-\Delta\Delta CT}$ formula, as the most common methodology. This has been included in the discussion. Each gene target settings for qPCR are relayed within Table 2 and 3. Attached is the reference for the $2^{-\Delta\Delta CT}$ calculation. https://pubmed.ncbi.nlm.nih.gov/11846609/	Page 8 line 209-215.
13	Compare each part of the result with similar studies and give reason if concurrent or controversial	Thank you for the kind comment. We have extensively elaborated each gene tested in this study and discussed the results along with comparing it in the literature.	Page 8 line 216 – page 9 line 272
14	Explain the limitations and future directions	We thank you for the constructive criticism. The limitations and future directions of this study has been added in the last part of the discussion	Page 10 line 291-299
15	Elaborate the message given to clinicians based on outcome of this study	We have added the clinical significance section after the conclusion to help clinicians apply the results of our study.	Page 11 line 308-312

Lactobacillus reuteri Probiotic Consumption reduced Various Virulence Gene Expression in Dental Plaque of Fixed Orthodontic Subjects.

Abstract

Aims: The aim of this study was to determine the effect of consuming lozenges containing *L. reuteri* probiotic Prodentis lozenges on the expression of *BCR1, ACE2*, *fadA, aid1, gelE*, and *atlA* genes in dental biofilms of subjects using fixed orthodontic appliances.

Materials and Methods: Plaque samples (n = 20) obtained in a previous study were used in this research. Each subject consumed *L. reuteri* probiotic lozenges (2×10^8 CFU/ml) each day for 2 weeks. RNA was extracted from the samples and synthesized into cDNA. The expression of the gene transcription factors *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE* and *atlA* genes in biofilms of subjects who used fixed orthodontic appliances was detected using RT-qPCR (Real Time Quantitative Polymerase Chain Reaction)

Results: The expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes were decreased after consuming the L. reuteri probiotic lozenges for 2 weeks (p<0.05).

Conclusion: Consuming *L. reuteri* probiotic lozenges would affect the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* in plaque from patients using fixed orthodontic appliances. By reducing the expression of the virulence genes, bacterial number would be reduced and biofilm production can also be reduced.

Clinical significance: Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression. Routine consumption of probiotic lozenges can help reduce potential bacterial infection and increase the oral health of patients using fixed orthodontic appliances.

Keywords: biofilm, gene expression, *Lactobacillus reuteri*, orthodontic, probiotic

Introduction:

Orthodontic treatment is common today in the community. Among adults and children, orthodontic treatment may be undertaken for dental care or esthetic reasons.[1] Orthodontic treatment using fixed appliances aims to ensure proper occlusion and esthetic function, with appropriate tooth movement. Fixed orthodontic treatment can lead to changes in the oral environment and oral flora composition and

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35 an increase in the amount of plaque due to difficulty in maintaining oral hygiene.[2-5] In
36 addition, excess composite around the base of the bracket that used in orthodontic
37 treatment is an important factor that can cause plaque accumulation due to the
38 presence of rough surfaces and cracks on the enamel composite surfaces.[3–5] Biofilm
39 accumulation on teeth and soft tissues in the oral cavity can lead to caries, gingivitis,
40 and periodontitis.[6,7]

41 *Fusobacterium nucleatum* is the dominant bacterial species and plays an
42 important role in the formation of dental biofilms and periodontal tissue disease. In the
43 formation of biofilms, *F. nucleatum* being a "bridging" or "linking" organism between
44 initial bacterial colonization and final bacterial colonization, which are unable to bind
45 to each other directly. *F. nucleatum* can also co-aggregate with various microbial
46 species in the oral cavity.[8–10] *F. nucleatum* encodes several adhesion genes involved
47 in interspecies interactions, including fusobacterium adhesion A (*fadA*), fusobacterial
48 outer membrane protein A (*fomA*), *radD* (an arginine-inhibitable adhesin), and
49 adherence inducing determinant gene 1 (*aid1*).[8,11–14] Fusobacterium adhesin A (FadA)
50 is known to be involved in *F. nucleatum* invasion and adhesion to host cells and is
51 highly conserved among oral Fusobacterium species.[8,15,16] FadA has been identified
52 has a major virulence factor in *F. nucleatum* in interspecies interactions with
53 *Streptococcus* mediated by *radD,* as it increases the binding specificity of *F. nucleatum*
54 to other microbial species.[14] The arginine-inhibitable adhesion *radD* is required by *F.*
55 *nucleatum* for co-adherence with various species of gram-positive bacteria, such as
56 streptococci (early colonizers), and fungal species, such as *Candida*.[11,12,17]

57 *Enterococcus faecalis* (*E. faecalis*) is associated with chronic periodontitis and
58 chronic apical periodontitis in failed root canal treatment.[18] *E. faecalis* is a gram-
59 positive aerobic bacterium. The severity of *E. faecalis* infection depends on the
60 immune response and virulence factors, which can exacerbate infection and play a
61 role in increasing biofilm formation.[19] There are several genes associated with *E.*
62 *faecalis* biofilm formation, including gelatinase (*gelE*) and autolysin (*atlA)*.[20] GelE in *E.*
63 *faecalis* plaque or saliva isolates showed resistance to antibiotics and high biofilm
64 formation ability.[21] atlA is the main peptidoglycan hydrolase or autolysin of *E.*
65 *faecalis.*[22] AtlA plays a role in the biofilm maturation stage during which extracellular
66 DNA (eDNA) is released and contributes to biofilm attachment and stability.[23,24]

67 An increase in the number of colonies of microorganisms also increased, one
68 of which was *Candida albicans*, which causes infections of oral mucosa.[25,26] *C.*

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69 *albicans* has a protein in the form of an adhesive that mediates other microorganisms 70 to adhere to abiotic and host surfaces to form biofilms.[27] Several *C. albicans* gene 71 transcription factors, including biofilm and cell wall regulator 1 (*BCR1*) and angiotensin 72 converting enzyme 2 (*ACE2*), play a role in the formation of biofilms. BCR1 acts as a 73 major regulator of *C. albicans* biofilm formation.[28] The ACE2 transcription factor plays 74 a role in fungal adherence, biofilm formation, and hyphal morphogenesis. In addition, 75 ACE2 plays a role in regulating the expression of genes involved in cell wall separation 76 and metabolism.[29] As shown in previous research, ACE2 is required for filamentation, 77 and it can increase the number of pseudohyphae cells at the time of biofilm 78 formation.[30]

79 Biofilm formation plays a role in increasing antibiotic resistance in bacterial 80 cells. Therefore, an effective therapy is needed to prevent biofilm formation. The use 81 of probiotics has been suggested as a promising approach to prevent and treat 82 microbial diseases and biofilm activity in the oral cavity.[31,32] Several studies have 83 proven that the use of probiotics has oral cavity health benefits, such as preventing 84 caries and periodontal disease.[32,33] One commercial probiotic proven to be beneficial 85 for oral health is *Lactobacillus reuteri*. The antimicrobial activity of *L. reuteri* inhibits 86 colonization by pathogenic microbes and interacts the inhibition directly with host 87 cells.[34] *L. reuteri* also inhibits the growth of *C. albicans* and *E. faecalis* biofilms.[35,36] 88 However, no studies have investigated the effect of the probiotic *L. reuteri* on the 89 expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in dental plaque biofilms 90 found in patient's oral environment with fixed orthodontic appliances. By analyzing the 91 virulence genes found in several pathogenic bacteria, we can hypothesize that the 92 downregulation of these genes will lead to less biofilm production and healthier oral 93 health conditions during the duration of fixed orthodontic appliances usage. Thus, to 94 bridge the knowledge gap, the aim of this study was to determine the effect of 95 consuming lozenges containing the probiotic *L. reuteri* on the expression of *BCR1*, 96 *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes in biofilms from subjects using fixed 97 orthodontic appliances.

98

99 **Methods:**

100 **Sample Collection**

101 Subjects undergoing fixed orthodontic therapy were enrolled in this open-label 102 prospective clinical trial. Ethical approval for the study was granted by the Institutional 103 Review Board of the Faculty of Dentistry, Universitas Indonesia (approval number: 104 100,701,020). The inclusion criteria specified participants aged 18 years or older who 105 had been undergoing orthodontic treatment with fixed appliances for a minimum 106 duration of one year and had not consumed probiotics or antibiotics in the preceding 107 three months. The fixed orthodontic appliances used by the subjects are conventional 108 metal braces fixed appliance. Exclusion criteria encompassed individuals with 109 systemic conditions such as hypertension or diabetes, those on systemic medications 110 including antihypertensives, analgesics, hormonal therapies, sedatives, or anti-seizure 111 medications, as well as individuals presenting with severe periodontal disease or 112 known allergies to probiotics.

113 The study utilized *L. reuteri* Prodentis lozenges, obtained from BioGaia 114 (Stockholm, Sweden). Each lozenge (800 mg) contained a minimum of 2×10^8 live 115 *Limosilactobacillus reuteri* Prodentis (previously classified as *Lactobacillus reuteri*). 116 This food supplement, specifically formulated for oral health, included a proprietary 117 combination of the *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289 strains, which 118 are recognized for their potential to support and maintain oral health.

119 To standardize oral hygiene practices, all participants were provided with a 120 standardized toothbrush and toothpaste for use throughout the study period. 121 Participants received oral hygiene instructions and were instructed to brush their teeth 122 twice daily. Each participant was administered one probiotic lozenge containing 123 *Limosilactobacillus reuteri* daily for 14 days, to be taken once per day after morning 124 toothbrushing and prior to breakfast. Plaque samples were collected from participants 125 on the day of enrolment, prior to the initiation of probiotic lozenge consumption, and 126 again on the 14th day following the completion of the probiotic regimen.

127 Before sample collection, mandatory rapid antigen test for SARS-CoV-2 128 detection had to be taken by the subjects in response to the COVID-19 pandemic (as 129 of 30 September 2020 when collecting the samples) and the test result had to be 130 negative for the subject partaking in the study. In brief, the participants were instructed 131 not to eat or drink anything two hours prior to collection of sample. Samples were 132 collected using sterile cotton buds and swabbed from 133 buccal/mesial/distal/lingual/occlusal surfaces of the index teeth of the subjects. The

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134 plaque samples were stored in sterile falcon tubes with 5 mL of phosphate-buffered
135 saline (PBS).

136 Furthermore, clinical data, including the Oral Hygiene Index-Simplified (OHI-S)
137 score and the Papilla Bleeding Index (PBI), were recorded at each visit. Previous
138 studies have demonstrated that a 14-day consumption of *Limosilactobacillus reuteri*
139 *Prodentis* significantly reduces clinical parameters such as the Periodontal Index (PI),
140 OHI-S, and Gingival Index (GI) in healthy individuals compared to a placebo
141 control.[37,38] Overall, twenty subjects had enrolled and fulfilled all requirements and
142 inclusion criteria. Plaque samples from the subjects were continued unto the following
143 downstream analysis.

144

145 **RNA Extraction, cDNA Synthesis and Quantification**

146 RNA from the sample was extracted using TRIzol reagent methodology as
147 instructed by the manufacturer (Thermo Fisher, Waltham, MA). The extracted RNA
148 was synthesized into cDNA using ReverTra Ace™ qPCR RT Master Mix with gDNA
149 Remover (Toyobo, Japan). The mixture for cDNA synthesis is described in Table 1.
150 The cDNA was then quantified using an Invitrogen™ Qubit 3.0 Fluorometer (Thermo
151 Fisher Scientific, Waltham, MA). The cDNA was stored at -20°C for storage of directly
152 used for downstream analysis.

153

154 **qPCR Analysis**

155 Amplification and detection by qPCR (Applied Biosystems, Waltham, MA) were
156 performed. The components of the qPCR Master Mix are listed in Table 2. Using a
157 specific kit, namely HOT FIREpol EvaGreen® qPCR Mix (Solis Biodyne, Tartu,
158 Estonia) which was activated by incubation at 95°C for 10 minutes. This was followed
159 by 40 cycles of denaturation at 95°C for 10 seconds, annealing temperature at
160 60−65°C (Table 3), and elongation at 72°C for 20 seconds. For templates longer than
161 150 bp, the annealing and elongation times were extended to 30 seconds. Actin gene
162 encoding as the housekeeping gene was used for normalization purposes. qPCR was
163 performed on cDNA. qPCR was performed using the primers listed in Table 3.

164

165 **Data Analysis and Outcome Analysis**

166 The data were analysed using the Shapiro−Wilk normality test ($p > 0.05$). For
167 data with a normal distribution, a paired *t*-test was applied. ($p < 0.05$). The software

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168 used for the analysis is Statistical Package for the Social Sciences (SPSS) version 27
169 (IBM, Armonk, NY). The outcome analysis are the quantitative data presented from
170 the RT-qPCR instrument. This data can then be correlated with previous study that
171 also conducted such research to ensure the validity of this data.
172

173 **Results:**

174 Based on the results of the qPCR test, the expression of *BCR1* (Graph 1A and
175 1B), *ACE2* (Graph 2A and 2B), *fadA* (Graph 3A and 3B), *aid1* (Graph 4A and 4B), *gelE*
176 (Graph 5A and 5B), and *atlA* (Graph 6A and 6B), on average, were decreased when
177 comparing the day-0 prior to probiotic consumption and day-14 after the subjects
178 consumed the probiotic *L. reuteri*.

179 As shown by the results of the Shapiro−Wilk normality test, all the data were
180 normally distributed ($p > 0.05$). The data were analysed using a paired *t*-test, with a
181 significance level of $p < 0.05$. The results of the paired *t*-test revealed a significant
182 difference in the comparison of the *BCR1* and *ACE2* gene expression data. As shown
183 in Table 4, there was a significant difference ($p < 0.05$) in the expression of *BCR1,*
184 *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes after consuming the probiotic *L. reuteri*.

185

186 **Discussion**:

187 The use of fixed orthodontic appliances often causes poor oral hygiene, thus
188 facilitating microorganism accumulation and various pathological conditions in the oral
189 cavity, such as fungal infections.[39] A previous study revealed an increase in *Candida*
190 in saliva after using fixed orthodontic appliances. The authors attributed this to the
191 design of fixed orthodontic appliances, which creates a space for the retention of food
192 waste.[40] Thus, patients must be instructed about good oral hygiene practices after
193 orthodontic treatment. [38] Fixed orthodontic appliances also induce changes in buffer
194 capacity, salivary flow rates, and acidity (pH), leading to plaque accumulation and an
195 increase in caries and periodontal disease.[41-43] Based on this information, we
196 attempted this study to prove the benefits of consuming probiotics lozenges as a
197 supplement for patients using fixed orthodontic appliances.

198 Biofilm formation is an important virulence factor of *F. nucleatum* due to its higher
199 resistance to host defence or antibacterial agents compared to planktonic cells.[44]
200 Several studies detected increased numbers of *Porphyromonas gingivalis, F.*
201 *nucleatum, P. intermedia,* and *Tannerella forsythia* after the use of fixed orthodontic
202 appliances.[15,45,46] They also reported that *F. nucleatum* increased the risk of
203 periodontitis in orthodontic patients due to a conducive environment for anaerobic
204 bacteria.[15,45,46] Biofilm formation is also a contributing factor to *E. faecalis* colonization
205 and infection. Biofilms develop through various processes by which bacteria adhere

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206 to surfaces, decompose complex matrices, and develop into bacterial colonies, which
207 adhere to surfaces.[47]

208 In this study, we used the qPCR method and $2^{-\Delta\Delta CT}$ formula to calculate target
209 gene expression. As shown by the results, the probiotic *L. reuteri* affected the
210 expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes and the formation of *C.*
211 *albicans, F. nucleatum*, and *E. faecalis* biofilms. Based on the Shapiro−Wilk normality
212 test, all the data were normally distributed, with $p > 0.05$. The paired *t*-test results
213 revealed significant differences in the expression *of BCR1, ACE2, fadA*, *aid1*, *gelE*,
214 and *atlA* genes (all $p < 0.05$).

215 Based on the results of this study, the probiotic *L. reuteri* significantly
216 downregulated the transcription of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes. In
217 gene expression, the process of translating genetic information in the form of
218 sequence of bases of DNA or RNA into proteins.[48] Through gene expression
219 measurement, it is possible to assess qualitatively and quantitatively the effect of a
220 treatment, such as the administration of a drug compound.[49] Gene expression in
221 microorganisms is involved in regulating cell-cell communication, carbohydrate
222 metabolism, adherence, and adaptation to the surrounding environment. A decrease
223 in the expression of specific genes can reduce microorganism colonization and
224 microorganism numbers.[50]

225 *BCR1*, a major gene transcription factor, produces an adhesin protein, which
226 facilitates *C. albicans* attachment to mucosal surfaces, which is a critical stage of
227 infection.[51] Deletion of *BCR1* eliminates *C. albicans* gene function, resulting in a
228 decrease in biofilm formation.[52] This was supported by the results of the present study,
229 which revealed a statistically significant decrease in *BCR1* gene expression after
230 consuming the probiotic.

231 The expression of *ACE2* also decreased based on the results of the statistical
232 tests. With deletion of *ACE2*, *C. albicans* is unable to form hyphal cells, and thus
233 biofilm formation is inhibited.[30] An earlier in vitro study showed that probiotics have
234 antifungal effects against *C. albicans* in the oral cavity. Regular use of probiotics
235 helped to inhibit *Candida* biofilms and reduced *Candida* colonization in the oral cavity,
236 thereby reducing the possibility of candidiasis infection.[53]

237 FadA protein is the main *F. nucleatum* virulence factor and mediates microbial
238 attachment and colonization.[8] Based on the results of this study, the probiotic *L. reuteri*

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239 appears to influence the pathogenicity of *F. nucleatum* adhesion molecules and
240 colonization and affect biofilm formation through decreased expression of the *fadA*
241 gene.[35] Various *F. nucleatum* adhesins mediate adhesion and aggregation and
242 function as coaggregation intermediaries in the formation and maturation of dental
243 biofilms.[54] The interaction of Fusobacterium with other species is largely mediated by
244 the adhesin genes *radD* and *aid1*.[55] The *aid1* gene plays a role in interspecies
245 interactions, colonization, and aggregation of *F. nucleatum*. In a previous study,
246 inactivation of the *aid1* gene decreased the ability of *F. nucleatum* to aggregate,
247 especially with *Streptococcus* spp. or *E. faecalis*.[14] As shown in earlier studies,
248 probiotics can affect the expression of genes involved in cell adhesion, quorum
249 sensing (QS), virulence factors, and biofilm formation.[56,57]

250 The *E. faecalis gelE* gene has the ability to hydrolyse gelatine, collagen, fibrin,
251 and other peptides.[58] Gene *gelE* is a virulence factor in infection formation through
252 bacterial attachment and biofilm formation.[22] In *E. faecalis*, biofilm formation is
253 regulated by QS, where Fsr regulates the expression of the *gelE* gene.[59] Fsr regulates
254 the formation of *E. faecalis* biofilms through its product *gelE* and serine proteases.[60]
255 QS is a molecular mechanism by which bacterial cells communicate with each other
256 via signalling molecules in biofilms. If the protease encoded by a signalling factor is
257 decreased, communication between bacteria and biofilm formation will be disrupted.[57]
258 GelE can also activate *atlA*, which is responsible for eDNA release at the biofilm
259 maturation stage.[61]

260 AtlA is involved in the hydrolysis of peptidoglycan, which plays an important role
261 in separating cells division after replication.[22] AtlA plays a role in the biofilm maturation
262 stage of *E. faecalis*, during which eDNA is released and contributes to biofilm
263 attachment and stability, biofilm defects in primary attachment, and decreased biofilm
264 production.[20,23] This study focused on the probiotic *L. reuteri*, which has ability to
265 secrete antimicrobial substances and compete with oral pathogens for adhesion to
266 mucosa. In addition, *L. reuteri* can adapt and change the pH of the surrounding
267 environment, thereby inhibiting the growth of oral pathogens.[62] The antimicrobial
268 substances secreted by *L. reuteri* are reuterin and reutericycline.[63] Reuterin and
269 reutericycline are broad-spectrum antimicrobial agents that are effective against gram-
270 positive and negative bacteria, fungi, and protozoa by inhibiting microbial DNA
271 synthesis.[35,41]

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272 Many dental and oral health care products used daily now include probiotics. The
273 use of probiotics is increasing due to their advantages over chemical agents, namely
274 reducing the risk of antibiotic resistance.[64] Probiotics work by modulating the immune
275 system, producing antimicrobial substances, and inhibiting certain pathogenic
276 organisms by interfering with adhesion, colonization, and biofilm formation. They
277 inhibit the growth of pathogens via the production of various substances, such as lactic
278 acid and acetic acid, which penetrate the bacterial cell membrane and lower the
279 cytoplasmic pH of pathogenic bacteria. Hydrogen peroxide and bacteriocin can
280 destroy the cell membrane of pathogenic bacteria and inhibit the synthesis of
281 pathogenic DNA.[32,33,35,65] Based on the results of this study, 2 weeks of daily
282 consumption of the probiotic *L. reuteri* affected the process of biofilm formation by
283 downregulating the expression of *BCR1*, *ACE2, fadA, aid1, gelE,* and *atlA g*enes,
284 which function as adherent regulators and regulators of hyphae formation in biofilm
285 formation. Many previous studies demonstrated that the addition of probiotics to dental
286 and oral health care products. Probiotic can reduced pathogenic microorganisms in
287 plaque samples from patients using fixed orthodontic appliances.[41,64,66–68] Therefore,
288 it can be stated that the probiotic *L. reuteri* has good ability as an additional treatment
289 for dental and oral health in patients using fixed orthodontic appliances.

290 The limitation of this study is the small sample size and the fact that the sampling
291 had to be done during COVID-19 pandemic. Although the sample size is small, the
292 result of this study can still provide concise and significant result. In the future, there
293 should be larger sample size for conducting this research so the result can more
294 accurately represents the actual population. Daily consumption of probiotic lozenges
295 duration can also be increased for more precise and accurate results. On the other
296 hand, consumption of probiotic lozenges research can also be conducted for other
297 aspect of oral health diseases to promote the functionality and health-inducing aspect
298 of probiotic lozenges, such as antiinflammation.
299

300 **Conclusion:**

301 Within the limitation of this study, it can be concluded that the probiotic *L. reuteri*
302 influences the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in biofilm
303 formation. By reducing the expression of those genes, the probiotic *L. reuteri* can

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304 reduce biofilm formation such as dental plaque in patients using fixed orthodontic
305 appliances.
306
307 **Clinical significance:** Consumption of probiotic lozenges were confirmed to reduce
308 bacterial and fungal biofilm, as proven by the reduction of virulence gene expression,
309 hence, helping increasing oral health of consumer. The results of this study help
310 clinicians provide probiotic lozenges for patients to promote and maintain their oral
311 health.
312
313
314 **List of abbreviations:**

315 °C : Celcius degree
316 ACE2 : Angiotensin Converting Enzyme 2
317 aid1 : adherence inducing determinant gene 1
318 atlA : autolysin
319 BCR1 : Biofilm and Cell wall Regulator 1
320 cDNA : copy Deoxyribonucleic Acid
321 CFU/mL : Colony forming unit per mililiter
322 DNA : Deoxyribonucleic Acid
323 eDNA : extracellular Deoxyribonucleic Acid
324 fadA : fusobacterium adhesion A
325 gelE : gelatinase
326 radD : arginine-inhibitable adhesin
327 RT-qPCR : Quantitative Polymerase Chain Reaction
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References:

1. Sihotang BM, Panjaitan M, Sitanggang JM, Putri A, Harahap FR. Perbedaan oral hygiene pemakaian pesawat ortodonti cekat dan pesawat ortodonti lepasan pada pasien klinik spesialis RS Herna. Jurnal Prima Medika Sains. 2020 Mar 15;2(1):1–5.
2. Gkantidis N, Christou P, Topouzelis N. The orthodontic-periodontic interrelationship in integrated treatment challenges: a systematic review. J Oral Rehabil. 2010 May 1;37(5):377–90.
3. Oktaviani V, Santoso O. Perbedaan Indeks Higiene Oral dan pH Plak Kelompok Pemakai dan Bukan Pemakai Pesawat Ortodonti Cekat. 2016;5(1):8.
4. Bowen WH, Burne RA, Wu H, Koo H. Oral Biofilms: Pathogens, Matrix and Polymicrobial Interactions in Microenvironments. Trends Microbiol. 2018 Mar;26(3):229–42.
5. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence. 2011;2(5):435–44.
6. Verrusio C, Iorio-Siciliano V, Blasi A, Leuci S, Adamo D, Nicolò M. The effect of orthodontic treatment on periodontal tissue inflammation: A systematic review. Quintessence Int. 2018;49(1):69–77.
7. Perkowski K, Baltaza W, Conn DB, Marczyńska-Stolarek M, Chomicz L. Examination of oral biofilm microbiota in patients using fixed orthodontic appliances in order to prevent risk factors for health complications. Ann Agric Environ Med. 2019 Jun 17;26(2):231–5.
8. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. Curr Opin Microbiol. 2015 Feb;0:141–7.
9. Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS. Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol. 2003 Feb;11(2):94–100.
10. Thurnheer T, Karygianni L, Flury M, Belibasakis GN. Fusobacterium Species and Subspecies Differentially Affect the Composition and Architecture of Supra- and Subgingival Biofilms Models. Front Microbiol. 2019 Jul 30;10:1716.
11. Chew J, Zilm PS, Fuss JM, Gully NJ. A proteomic investigation of Fusobacterium nucleatum alkaline-induced biofilms. BMC Microbiology. 2012 Sep 3;12(1):189.
12. Kaplan CW, Lux R, Haake SK, Shi W. The Fusobacterium nucleatum Outer Membrane Protein RadD Is an Arginine-Inhibitable Adhesin Required for Inter-Species Adherence and the Structured Architecture of Multi-Species Biofilm. Mol Microbiol. 2009 Jan;71(1):35–47.
13. Cochrane K, Robinson AV, Holt RA, Allen-Vercoe E. A survey of Fusobacterium nucleatum genes modulated by host cell infection. Microb Genom. 2020 Feb;6(2).
14. Kaplan A, Kaplan CW, He X, McHardy I, Shi W, Lux R. Characterization of aid1, a novel gene involved in Fusobacterium nucleatum interspecies interactions. Microb Ecol. 2014 Aug;68(2):379–87.
15. Liu P, Liu Y, Wang J, Guo Y, Zhang Y, Xiao S. Detection of Fusobacterium Nucleatum and fadA Adhesin Gene in Patients with Orthodontic Gingivitis and Non-Orthodontic Periodontal Inflammation. PLOS ONE. 2014 Jan 9;9(1):e85280.

375 16. Brennan CA, Garrett WS. Fusobacterium nucleatum - symbiont, opportunist and
376 oncobacterium. Nat Rev Microbiol. 2019 Mar;17(3):156–66.
377 17. Wu T, Cen L, Kaplan C, Zhou X, Lux R, Shi W, et al. Cellular Components
378 Mediating Coadherence of Candida albicans and Fusobacterium nucleatum. J
379 Dent Res. 2015 Oct;94(10):1432–8.
380 18. Wang Q-Q, Zhang C-F, Chu C-H, Zhu X-F. Prevalence of Enterococcus faecalis
381 in saliva and filled root canals of teeth associated with apical periodontitis. Int J
382 Oral Sci. 2012 Apr;4(1):19–23.
383 19. Najafi K, Gholizadeh P, Tanomand A, Ahangarzadeh Rezaee M, Mahmood S,
384 Asgharzadeh M, et al. Oral Cavity infection by Enterococcus Faecalis: virulence
385 factors and pathogenesis. Reviews in Medical Microbiology. 2019 Jan 2;30.
386 20. Ch'ng J-H, Chong KKL, Lam LN, Wong JJ, Kline KA. Biofilm-associated infection
387 by enterococci. Nat Rev Microbiol. 2019 Jan;17(2):82–94.
388 21. Anderson AC, Jonas D, Huber I, Karygianni L, Wölber J, Hellwig E, et al.
389 Enterococcus faecalis from Food, Clinical Specimens, and Oral Sites:
390 Prevalence of Virulence Factors in Association with Biofilm Formation. Front
391 Microbiol. 2016 Jan 11;6:1534.
392 22. Stinemetz EK, Gao P, Pinkston KL, Montealegre MC, Murray BE, Harvey BR.
393 Processing of the major autolysin of E. faecalis, AtlA, by the zinc-
394 metalloprotease, GelE, impacts AtlA septal localization and cell separation.
395 PLOS ONE. 2017 Oct 19;12(10):e0186706.
396 23. Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, et al. Contribution of
397 Autolysin and Sortase A during Enterococcus faecalis DNA-Dependent Biofilm
398 Development. Infect Immun. 2009 Sep;77(9):3626–38.
399 24. Paganelli FL, Willems RJL, Jansen P, Hendrickx A, Zhang X, Bonten MJM, et al.
400 Enterococcus faecium Biofilm Formation: Identification of Major Autolysin
401 AtlAEfm, Associated Acm Surface Localization, and AtlAEfm-Independent
402 Extracellular DNA Release. mBio. 2013 Apr 16;4(2):e00154-13.
403 25. Putri MH, Herijulianti E. Ilmu Pencegahan Penyakit Jaringan Keras dan Jaringan
404 Pendukung Gigi | OPAC Integrasi | Online Public Access Catalog | Universitas
405 Gadjah Mada. RGC; 2010.
406 26. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent
407 public health problem. Clin Microbiol Rev. 2007;20(1):133–63.
408 27. Garcia MC, Lee JT, Ramsook CB, Alsteens D, Dufrêne YF, Lipke PN. A Role for
409 Amyloid in Cell Aggregation and Biofilm Formation. PLOS ONE.
410 2011;6(3):e17632.
411 28. Nobile CJ, Mitchell AP. Regulation of Cell-Surface Genes and Biofilm Formation
412 by the C. albicans Transcription Factor Bcr1p. Current Biology. 2005 Jun
413 21;15(12):1150–5.
414 29. Mulhern SM, Logue ME, Butler G. Candida albicans Transcription Factor Ace2
415 Regulates Metabolism and Is Required for Filamentation in Hypoxic Conditions.
416 Eukaryot Cell. 2006 Dec;5(12):2001–13.
417 30. Saputo S, Kumar A, Krysan DJ. Efg1 Directly Regulates ACE2 Expression To
418 Mediate Cross Talk between the cAMP/PKA and RAM Pathways during Candida
419 albicans Morphogenesis. Eukaryot Cell. 2014 Sep;13(9):1169–80.

420 31. Widyarman AS, Bachtiar EW, Bachtiar BM, Seneviratne CJ. Inhibitory effect of
421 probiotic lactobacilli against Streptococcus mutans and Porphyromonas
422 gingivalis biofilms. Scientific Dental Journal. 2019 May 1;3(2):50.
423 32. Widyarman AS, Ramajayanti T, Theodorea CF. Indonesian Strain of Probiotic
424 Reduces the Initial Biofilm Colonization. The Open Dentistry Journal [Internet].
425 2020 Nov 13 [cited 2022 Feb 13];14(1). Available from:
426 https://opendentistryjournal.com/VOLUME/14/PAGE/544/FULLTEXT/
427 33. Widyarman AS, Theodorea CF. Novel Indigenous Probiotic Lactobacillus reuteri
428 Strain Produces Anti-biofilm Reuterin against Pathogenic Periodontal Bacteria.
429 Eur J Dent [Internet]. 2021 Jul 24 [cited 2022 Feb 13]; Available from:
430 http://www.thieme-connect.de/DOI/DOI?10.1055/s-0041-1731591
431 34. Mu Q, Tavella VJ, Luo XM. Role of Lactobacillus reuteri in Human Health and
432 Diseases. Frontiers in Microbiology [Internet]. 2018 [cited 2022 Feb 13];9.
433 Available from: https://www.frontiersin.org/article/10.3389/fmicb.2018.00757
434 35. Department of Microbiology, Faculty of Dentistry, Trisakti University, Jakarta,
435 11440 Indonesia, Sari Widyarman A, Kiky Elysia Lazaroni N, Undergraduate
436 Program, Faculty of Dentistry, Trisakti University, Jakarta, 11440 Indonesia.
437 Persistent Endodontics Pathogens Biofilm Inhibited by Lactobacillus reuteri
438 Indonesian Strain Lactobacillus reuteri Indonesian. J Dent Indones [Internet].
439 2019 Dec 31 [cited 2022 Feb 13];26(3). Available from:
440 https://scholarhub.ui.ac.id/jdi/vol26/iss3/7/
441 36. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of
442 probiotic and pathogen strains. Eur Food Res Technol. 2008;226(5):1065–73.
443 37. Twetman S, Derawi B, Keller M, et al. Short-term effect of chewing gums
444 containing probiotic Lactobacillus reuteri on the levels of inflammatory mediators
445 in gingival crevicular fluid. Acta Odontol Scand. 2009;67(1):19–24.
446 38. Krasse P, Carlsson B, Dahl C, et al. Decreased gum bleeding and reduced
447 gingivitis by the probiotic Lactobacillus reuteri. Swed Dent J. 2006;30(2):55–60.
448
449 39. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. J Oral
450 Microbiol [Internet]. 2011;3. Available from:
451 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3087208/
452 40. Khanpayeh E, Jafari AA, Tabatabaei Z. Comparison of salivary Candida profile
453 in patients with fixed and removable orthodontic appliances therapy. Iran J
454 Microbiol. 2014 Aug;6(4):263–8.
455 41. Widyarman AS, Hartono V, Marjani LI, Irawan D, Luthfi L, Bachtiar BM.
456 Lactobacillus reuteri containing Probiotic Lozenges Consumption Reduces
457 Streptococcus mutans, Streptococcus sobrinus, Porphyromonas gingivalis, and
458 Aggregatibacter actinomycetemcomitans in Orthodontic Patients. Journal of
459 International Dental and Medical Research. 2018 Jan 1;11(2):628–33.
460 42. Restrepo M, Bussaneli DG, Jeremias F, Cordeiro RCL, Raveli DB, Magalhães
461 AC, et al. Control of White Spot Lesions with Use of Fluoride Varnish or
462 Chlorhexidine Gel During Orthodontic Treatment A Randomized Clinical Trial. J
463 Clin Pediatr Dent. 2016;40(4):274–80.

JAYPEE

43. Lu H, Tang H, Zhou T, Kang N. Assessment of the periodontal health status in patients undergoing orthodontic treatment with fixed appliances and Invisalign system. Medicine (Baltimore). 2018 Mar 30;97(13):e0248.
44. Fitriasari N, Wahyuni IS. POTENSI PROBIOTIK DALAM TATALAKSANA ORAL CANDIDIASIS: Ulasan Sistematik. ODONTO : Dental Journal. 2021 Jul 12;8(1):34–44.
45. Guo L, Feng Y, Guo H-G, Liu B-W, Zhang Y. Consequences of orthodontic treatment in malocclusion patients: clinical and microbial effects in adults and children. BMC Oral Health. 2016 Oct 28;16:112.
46. Alqerban A. Efficacy of antimicrobial photodynamic and photobiomodulation therapy against Treponema denticola, fusobacterium nucleatum and human beta defensin-2 levels in patients with gingivitis undergoing fixed orthodontic treatment: A clinic-laboratory study. Photodiagnosis and Photodynamic Therapy. 2020 Mar 1;29:101659.
47. Willett JLE, Ji MM, Dunny GM. Exploiting biofilm phenotypes for functional characterization of hypothetical genes in Enterococcus faecalis. npj Biofilms Microbiomes. 2019 Sep 19;5(1):1–14.
48. Sinaga E. Regulasi Ekspresi Gen. Regulasi Ekspresi Gen. 2011;1–24.
49. Van Peer G, Mestdagh P, Vandesompele J. Accurate RT-qPCR gene expression analysis on cell culture lysates. Sci Rep. 2012 Jan 13;2:222.
50. Shemesh M, Tam A, Steinberg D 2007. Expression of biofilm-associated genes of Streptococcus mutans in response to glucose and sucrose. Journal of Medical Microbiology. 56(11):1528–35.
51. Nikoomanesh F, Department of medical mycology, Faculty of medical science, Tarbiat Modares University, Tehran, Iran, Roudbarmohammadi S, Department of medical mycology, Faculty of medical science, Tarbiat Modares University, Tehran, Iran, Roudbary M, Department of Medical Mycology and Parasitology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran, et al. Investigation of bcr1 Gene Expression in Candida albicans Isolates by RT-PCR Technique and its Impact on Biofilm Formation. iem. 2016 Jan 1;2(1):22–4.
52. Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP. Divergent Targets of Candida albicans Biofilm Regulator Bcr1 In Vitro and In Vivo. Eukaryotic Cell. 2012 Jul;11(7):896–904.
53. Matsubara VH, Bandara HMHN, Mayer MPA, Samaranayake LP. Probiotics as Antifungals in Mucosal Candidiasis. Clin Infect Dis. 2016;62(9):1143–53.
54. Li Q, Tan L, Wang H, Kou Y, Shi X, Zhang S, et al. Fusobacterium nucleatum Interaction with Pseudomonas aeruginosa Induces Biofilm-Associated Antibiotic Tolerance via Fusobacterium Adhesin A. ACS Infect Dis. 2020 Jul 10;6(7):1686–96.
55. Kaplan A. Identification and Characterization of a Novel Lipoprotein in Fusobacterium nucleatum [Internet]. UCLA; 2015 [cited 2022 Feb 13]. Available from: https://escholarship.org/uc/item/3r90n33c
56. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402–8.

JAYPEE

509 57. Barzegari A, Kheyrolahzadeh K, Hosseiniyan Khatibi SM, Sharifi S, Memar MY, Zununi Vahed S. The Battle of Probiotics and Their Derivatives Against Biofilms. Infect Drug Resist. 2020 Feb 26;13:659–72.

512 58. Guneser MB, Eldeniz AU. The effect of gelatinase production of Enterococcus faecalis on adhesion to dentin after irrigation with various endodontic irrigants. Acta Biomater Odontol Scand. 2016 Nov 15;2(1):144–9.

515 59. Hashem YA, Amin HM, Essam TM, Yassin AS, Aziz RK. Biofilm formation in enterococci: genotype-phenotype correlations and inhibition by vancomycin. Sci Rep. 2017 Jul 18;7(1):5733.

518 60. Wang L, Dong M, Zheng J, Song Q, Yin W, Li J, et al. Relationship of biofilm formation and gelE gene expression in Enterococcus faecalis recovered from root canals in patients requiring endodontic retreatment. J Endod. 2011 May;37(5):631–6.

522 61. Ali L, Goraya MU, Arafat Y, Ajmal M, Chen J-L, Yu D. Molecular Mechanism of Quorum-Sensing in Enterococcus faecalis: Its Role in Virulence and Therapeutic Approaches. Int J Mol Sci. 2017 May 3;18(5):960.

525 62. Bonifait L. Probiotics for Oral Health: Myth or Reality? 2009;75(8):6.

526 63. Urbańska M, Szajewska H. The efficacy of Lactobacillus reuteri DSM 17938 in infants and children: a review of the current evidence. Eur J Pediatr. 2014;173(10):1327–37.

529 64. Benic GZ, Farella M, Morgan XC, Viswam J, Heng NC, Cannon RD, et al. Oral probiotics reduce halitosis in patients wearing orthodontic braces: a randomized, triple-blind, placebo-controlled trial. J Breath Res. 2019 May 31;13(3):036010.

532 65. Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A. Probiotic mechanisms of action. Ann Nutr Metab. 2012;61(2):160–74.

534 66. Widyarman AS, Yunita ST, Prasetyadi T. Consumption of Yogurt Containing Probiotic *Bifidobacterium lactis* Reduces *Streptococcus mutans* in Orthodontic Patients. Scientific Dental Journal. 2018 Jan 25;2(1):19–25.

537 67. Alp S, Baka ZM. Effects of probiotics on salivary *Streptecoccus mutans* and Lactobacillus levels in orthodontic patients. Am J Orthod Dentofacial Orthop. 2018 Oct;154(4):517–23.

540 68. Goyal N, Shamanna PU, Varughese ST, Abraham R, Antony B, Emmatty R, et al. Effects of amine fluoride and probiotic mouthwash on levels of *Porphyromonas gingivalis* in orthodontic patients: A randomized controlled trial. J Indian Soc Periodontol. 2019;23(4):339–44.

544 69. Ranjith K, Kalyana Chakravarthy S, Adicherla H, Sharma S, Shivaji S. Temporal Expression of Genes in Biofilm-Forming Ocular *Candida albicans* Isolated From Patients With Keratitis and Orbital Cellulitis. Investigative Ophthalmology & Visual Science. 2018 Jan 25;59(1):528–38.

548 70. Nailis H, Coenye T, Van Nieuwerburgh F, Deforce D, Nelis HJ. Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. BMC Mol Biol. 2006 Aug 4;7:25.

551 71. Vos M, Quince C, Pijl AS, Hollander M de, Kowalchuk GA. A Comparison of rpoB and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity. PLOS ONE. 2012 Feb 15;7(2):e30600.

JAYPEE

554 72. Zheng J-X, Wu Y, Lin Z-W, Pu Z-Y, Yao W-M, Chen Z, et al. Characteristics of
555 and Virulence Factors Associated with Biofilm Formation in Clinical
556 Enterococcus faecalis Isolates in China. Front Microbiol. 2017 Nov 24;8:2338

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560 **Figures and figure legends**

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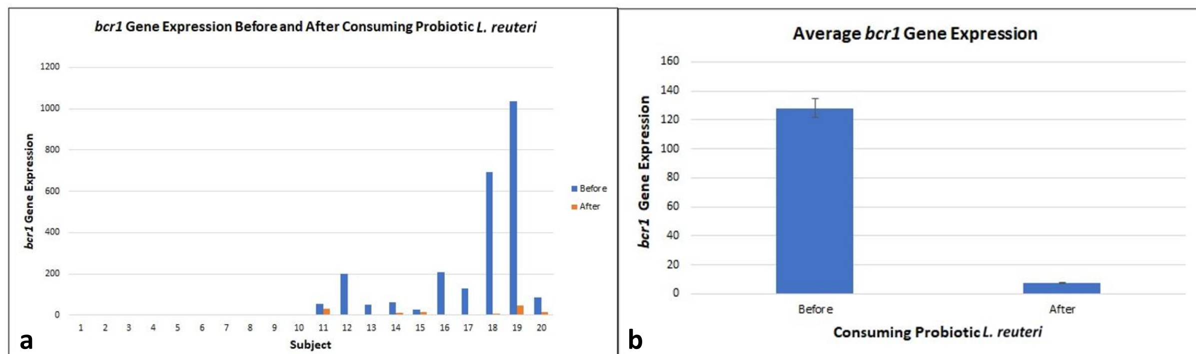

562
563 **Graph 1**. (**a**) A graph showing bcr1 gene expression in plaque samples *(N*= 20) as
564 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
565 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
566 *bcr1* gene expression in plaque samples as assessed by the RT-qPCR method and
567 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
568 lozenges.

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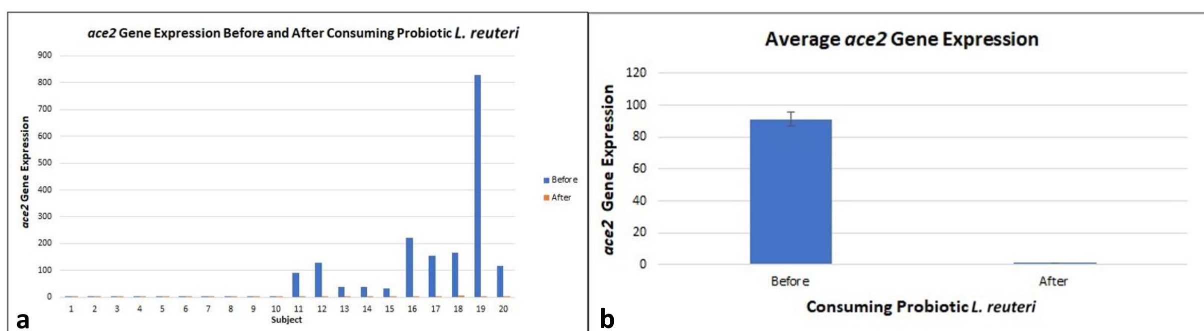

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572 **Graph 2**. (**a**) A graph showing ace2 gene expression in plaque samples *(N*= 20) as
573 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
574 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
575 *ace2* gene expression in plaque samples as assessed by the RT-qPCR method and
576 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
577 lozenges.

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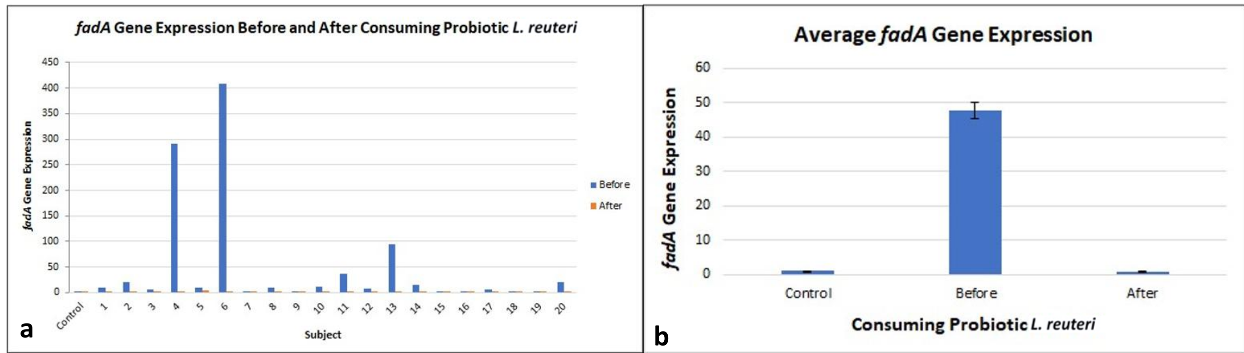
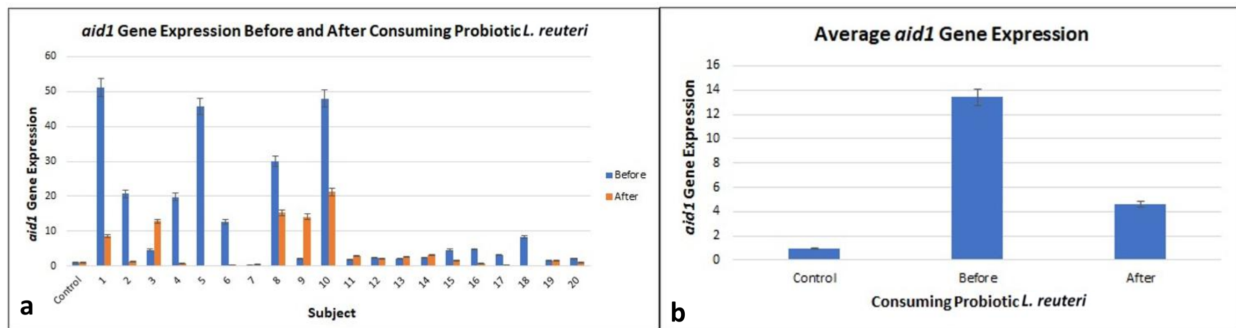
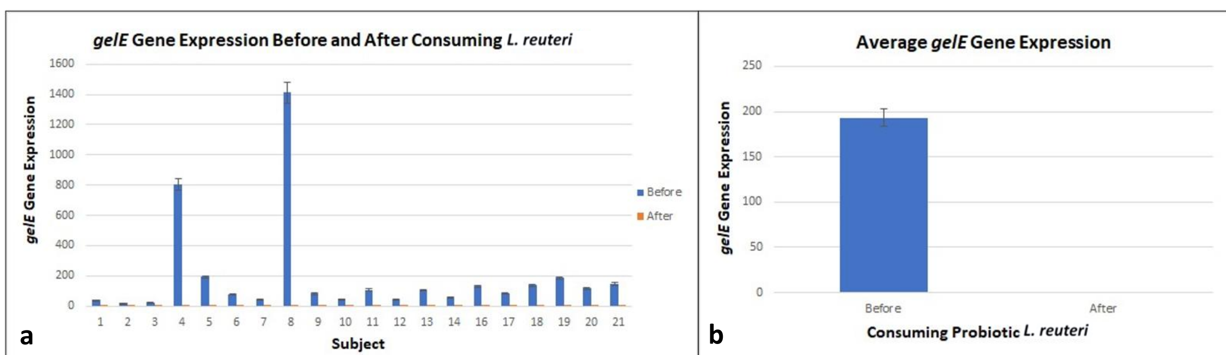


Graph 3. (**a**) A graph showing fadA gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of *fadA* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges.

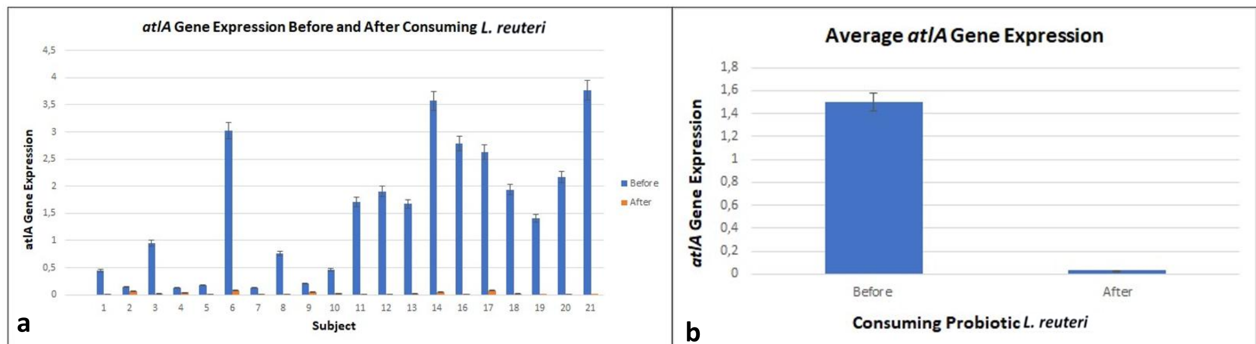


Graph 4. (**a**) A graph showing aid1 gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of *aid1* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges.



Graph 5. (**a**) A graph showing gelE gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of

gelE gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges.



Graph 6. (**a**) A graph showing atlA gene expression in plaque samples *(N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of *atlA* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges.

625 ## Tables and table legends

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627 **Table 1.** Reagent components for DNase I Reaction Solution

Component	**Volume**
4x DN *Master Mix*	2 mL
RNA *template*	0,5 pg – 0,5 mg
Nuclease-free Water	X mL
Total Volume	**8 mL**

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629 **Table 2.** Components of Master Mix RT-qPCR

Component	**Volume**
5 x HOT FIREPol EvaGreen® qPCR Mix Plus	4 mL
Primer *Forward*	1 mL
Primer *Reverse*	1 mL
DNA *template*	2 mL
NFW	12 mL
Total	**20 mL**

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633 **Table 3.** *Primer sequence* for RT-qPCR

Genes	*Primer sequence*	
BCR1[51]	*forward*: 5'-CTTCAGCAGCTTCATTAACACCTA-3' *reverse*: 5'-TCTTGGATCAGGTGTACTTTTCAA-3'	Initial denaturation of 95°C for 5 minutes; 40 Cycles of denaturation at 95°C for 1 minute and annealing at 58°C for 1 minute.
ACE2[69]	*forward* 5'-AGAATTGACCGTTGTCCGTGTAAG-3' *reverse*: 5'-AATGGGTGAATAAATCCCTCCCTAA-3'	Initial denaturation 95⁰C for 2 minutes; 40 Cycles of denaturation at 95°C for 30 seconds and annealing at 60°C for 1 minute.
Housekeeping gene C. albicans : *ACT1*[70]	*forward*: 5'-TTTCATCTTCTGTATCAGAGGAACTTATTT-3' *reverse*: 5'-ATGGGATGAATCATCAAACAAGAG-3'	Initial denaturation 95°C for 10 minutes; 40 Cycles of denaturing 95°C for 15 seconds and annealing 60°C for 1 minute

fadA[15]	*forward*: 5' -CAC AAG CTG ACG CTG CTA GA- 3' *reverse*: 5' -TTA CCA GCT CTT AAA GCT TG- 3'	Initial incubation for 4 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55.8°C for 30 seconds, and elongation at 72°C for 40 seconds and the final elongation for 6 min.[14]
aid1[14]	*forward:* 5' -TACAGGAG GTGCCGTAGCAG- 3' *reverse:* 5' -TTTTTGTTAATTCT CCAGCTCCA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.[13]
Housekeeping gene *F. nucleatum*: *rpoB*[71]	*forward:* 5'- GGYTWYGAAGTNCGHGACGTDCA - 3' *reverse:* 5'-TGACGYTGCATGTTBGMR CCCATMA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.

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gelE[60]	*forward:* 5’- CGGAACATACTGCCGGTTTAGA - 3’ *reverse:* 5’- TGGATTAGATGCACCCGAAAT - 3’	Initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 5 seconds, and annealing at 60°C for 30 seconds.
atlA[72]	*forward:* 5’- AATAATCAATCAGGAACGAATACG - 3’ *reverse:* 5’- GCCACACTAACACCGAAT -3’	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.
Housekeeping gene E. faecalis: rpoA[72]	*forward:* 5’- GTGAAACCTGGTCGTGGCTA - 3’ *reverse:* 5’- CGACGAACGGGTGTGTAGAT- 3’	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.

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6. Bukti konfirmasi submit revisi, respon kepada reviewer, dan artikel yang diresubmit

12 Mei 2025

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JCDP_24_408_R4 has been sent for review

1 message

noreply@jaypeejournals.com <noreply@jaypeejournals.com> Mon, May 12, 2025 at 2:39 PM
To: armeliasari@trisakti.ac.id, joko.k@trisakti.ac.id, sarasafirah@gmail.com, litayanas@gmail.com, winnie.valentini@gmail.com
Cc: ayushi.goyal@jaypeebrothers.com

Dear Dr. Armelia Sari Widyarman,

The article **Lactobacillus reuteri Probiotic Consumption reduced Various Virulence Gene Expression in Dental Plaque of Fixed Orthodontic Subjects** submitted by you through Scriptor's Zone in Journal The Journal of Contemporary Dental Practice has passed the preliminary stage and has now been further sent to Editor/Reviewers for peer-review process.

Once the review is over, you will be informed of the Editorial decision via e-mail.

We thank you for your contribution to the journal and look forward for a continued support.

Thanking you

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No.	Reviewer's Comment	Author's Response	Location of Revision
1	Prepare a **short running title** for your manuscript to be used as header for each page. (Length: not more than 10 words)	We have prepared a short running title "Probiotic consumption reduced virulence gene expression from fixed orthodontic plaque." It has been inserted at the header for each page	Header
2	**Abstract**: In results section please mention p value	we have mentioned p value in abstract result section ($p<0.05$)	Page 1 line 18
4	Brief about the genes assessed in the study	Thank you for your kind comment. Each gene has been discussed briefly in the introduction.	page 1 line 41 to page 2 line 78
5	Elaborate inclusion and exclusion criteria. Here mention which type of fixed appliance were included in the study	the fixed appliances used in this study is conventional metal braces fixed appliance.	page 4 line 107-108
6	Explain if study groups were there ie control or only before and after comparison was done	the study groups were only before probiotic consumption and after 14-day of probiotic consumption	page 4 line 124-126.
7	Justify sample size calculation	twenty sample was determined due to this study being a pilot study. Attached below are the references that we used to justify the sample size.	Page 5 line 137-139

		• Julious SA (2005) Sample size of 12 per group rule of thumb for a pilot study. Pharm Stat 4:287–291. https://doi.org/10.1002/pst.185 • Johanson GA, Brooks GP (2010) Initial scale development: sample size for pilot studies. Educ Psychol Meas 70:394–400. https://doi.org/10.1177/0013164409355692	
8	Explain randomization and blinding method	this was an open-blinded study, as collection of participating subjects during the COVID-19 pandemic was proved to be a challenging matter. The subjects were subjected to COVID-19 rapid test prior to sample collection; therefore, it was not possible to blind the subjects from the treatment they were given.	Page 4 line 101-102
9	Explain inference of the study.	Thank you for the instructive criticism. we have displayed the results and inferred in general how probiotic consumption correlates with the reduction of gene expression of said pathogens	Page 7 line 171 – 181
10	Compare each part of the result with similar studies and give reason if concurrent or controversial	Thank you for the kind comment. We have extensively elaborated each gene tested in this study and discussed the results along with comparing it in the literature.	Page 7 line 195 – page 9 line 268
14	Explain the limitations and future directions	We thank you for the constructive criticism. The limitations and future directions of this study has been added in the last part of the discussion	Page 10 line 287-295

15	Elaborate the message given to clinicians based on outcome of this study	We have added the clinical significance section after the conclusion to help clinicians apply the results of our study.	Page 11 line 304-308

1 # *Lactobacillus reuteri* Probiotic Consumption reduced
2 # Various Virulence Gene Expression in Dental Plaque of
3 # Fixed Orthodontic Subjects.
4
5
6 ## Abstract

7 **Aims:** The aim of this study was to determine the effect of consuming lozenges
8 containing *L. reuteri* probiotic Prodentis lozenges on the expression of *BCR1, ACE2*,
9 *fadA, aid1, gelE*, and *atlA* genes in dental biofilms of subjects using fixed orthodontic
10 appliances.

11 **Materials and Methods:** Plaque samples (n = 20) obtained in a previous study were
12 used in this research. Each subject consumed *L. reuteri* probiotic lozenges (2 × 10^8
13 CFU/ml) each day for 2 weeks. RNA was extracted from the samples and synthesized
14 into cDNA. The expression of the gene transcription factors *BCR1*, *ACE2*, *fadA*, *aid1*,
15 *gelE* and *atlA* genes in biofilms of subjects who used fixed orthodontic appliances was
16 detected using RT-qPCR (Real Time Quantitative Polymerase Chain Reaction)

17 **Results:** The expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes were
18 decreased after consuming the L. reuteri probiotic lozenges for 2 weeks ($p<0.05$).

19 **Conclusion:** Consuming *L. reuteri* probiotic lozenges would affect the expression of
20 *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* in plaque from patients using fixed orthodontic
21 appliances. By reducing the expression of the virulence genes, bacterial number would
22 be reduced and biofilm production can also be reduced.

23 **Clinical significance:** Consumption of probiotic lozenges were confirmed to reduce
24 bacterial and fungal biofilm, as proven by the reduction of virulence gene expression.
25 Routine consumption of probiotic lozenges can help reduce potential bacterial
26 infection and increase the oral health of patients using fixed orthodontic appliances.

27 **Keywords:** biofilm, gene expression, *Lactobacillus reuteri*, orthodontic, probiotic
28

29 ## Introduction:

30 Orthodontic treatment is common today in the community. Among adults and
31 children, orthodontic treatment may be undertaken for dental care or esthetic
32 reasons.[1] Orthodontic treatment using fixed appliances aims to ensure proper
33 occlusion and esthetic function, with appropriate tooth movement. Fixed orthodontic
34 treatment can lead to changes in the oral environment and oral flora composition and

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35 an increase in the amount of plaque due to difficulty in maintaining oral hygiene.[2-5] In
36 addition, excess composite around the base of the bracket that used in orthodontic
37 treatment is an important factor that can cause plaque accumulation due to the
38 presence of rough surfaces and cracks on the enamel composite surfaces.[3–5] Biofilm
39 accumulation on teeth and soft tissues in the oral cavity can lead to caries, gingivitis,
40 and periodontitis.[6,7]

41 *Fusobacterium nucleatum* is the dominant bacterial species and plays an
42 important role in the formation of dental biofilms and periodontal tissue disease. In the
43 formation of biofilms, *F. nucleatum* being a "bridging" or "linking" organism between
44 initial bacterial colonization and final bacterial colonization, which are unable to bind
45 to each other directly. *F. nucleatum* can also co-aggregate with various microbial
46 species in the oral cavity.[8–10] *F. nucleatum* encodes several adhesion genes involved
47 in interspecies interactions, including fusobacterium adhesion A (*fadA*), fusobacterial
48 outer membrane protein A (*fomA*), *radD* (an arginine-inhibitable adhesin), and
49 adherence inducing determinant gene 1 (*aid1*).[8,11–14] Fusobacterium adhesin A (FadA)
50 is known to be involved in *F. nucleatum* invasion and adhesion to host cells and is
51 highly conserved among oral Fusobacterium species.[8,15,16] FadA has been identified
52 has a major virulence factor in *F. nucleatum* in interspecies interactions with
53 *Streptococcus* mediated by *radD,* as it increases the binding specificity of *F. nucleatum*
54 to other microbial species.[14] The arginine-inhibitable adhesion *radD* is required by *F.*
55 *nucleatum* for co-adherence with various species of gram-positive bacteria, such as
56 streptococci (early colonizers), and fungal species, such as *Candida*.[11,12,17]

57 *Enterococcus faecalis* (*E. faecalis*) is associated with chronic periodontitis and
58 chronic apical periodontitis in failed root canal treatment.[18] *E. faecalis* is a gram-
59 positive aerobic bacterium. The severity of *E. faecalis* infection depends on the
60 immune response and virulence factors, which can exacerbate infection and play a
61 role in increasing biofilm formation.[19] There are several genes associated with *E.*
62 *faecalis* biofilm formation, including gelatinase (*gelE*) and autolysin (*atlA)*.[20] GelE in *E.*
63 *faecalis* plaque or saliva isolates showed resistance to antibiotics and high biofilm
64 formation ability.[21] atlA is the main peptidoglycan hydrolase or autolysin of *E.*
65 *faecalis.*[22] AtlA plays a role in the biofilm maturation stage during which extracellular
66 DNA (eDNA) is released and contributes to biofilm attachment and stability.[23,24]

67 An increase in the number of colonies of microorganisms also increased, one
68 of which was *Candida albicans*, which causes infections of oral mucosa.[25,26] *C.*

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albicans has a protein in the form of an adhesive that mediates other microorganisms to adhere to abiotic and host surfaces to form biofilms.[27] Several *C. albicans* gene transcription factors, including biofilm and cell wall regulator 1 (*BCR1*) and angiotensin converting enzyme 2 (*ACE2*), play a role in the formation of biofilms. BCR1 acts as a major regulator of *C. albicans* biofilm formation.[28] The ACE2 transcription factor plays a role in fungal adherence, biofilm formation, and hyphal morphogenesis. In addition, ACE2 plays a role in regulating the expression of genes involved in cell wall separation and metabolism.[29] As shown in previous research, ACE2 is required for filamentation, and it can increase the number of pseudohyphae cells at the time of biofilm formation.[30]

Biofilm formation plays a role in increasing antibiotic resistance in bacterial cells. Therefore, an effective therapy is needed to prevent biofilm formation. The use of probiotics has been suggested as a promising approach to prevent and treat microbial diseases and biofilm activity in the oral cavity.[31,32] Several studies have proven that the use of probiotics has oral cavity health benefits, such as preventing caries and periodontal disease.[32,33] One commercial probiotic proven to be beneficial for oral health is *Lactobacillus reuteri*. The antimicrobial activity of *L. reuteri* inhibits colonization by pathogenic microbes and interacts the inhibition directly with host cells.[34] *L. reuteri* also inhibits the growth of *C. albicans* and *E. faecalis* biofilms.[35,36] However, no studies have investigated the effect of the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in dental plaque biofilms found in patient's oral environment with fixed orthodontic appliances. By analyzing the virulence genes found in several pathogenic bacteria, we can hypothesize that the downregulation of these genes will lead to less biofilm production and healthier oral health conditions during the duration of fixed orthodontic appliances usage. Thus, to bridge the knowledge gap, the aim of this study was to determine the effect of consuming lozenges containing the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes in biofilms from subjects using fixed orthodontic appliances.

Methods:

Sample Collection

101 Subjects undergoing fixed orthodontic therapy were enrolled in this open-label
102 prospective clinical trial. Ethical approval for the study was granted by the Institutional
103 Review Board of the Faculty of Dentistry, Universitas Indonesia (approval number:
104 100,701,020). The inclusion criteria specified participants aged 18 years or older who
105 had been undergoing orthodontic treatment with fixed appliances for a minimum
106 duration of one year and had not consumed probiotics or antibiotics in the preceding
107 three months. The fixed orthodontic appliances used by the subjects are conventional
108 metal braces fixed appliance. Exclusion criteria encompassed individuals with
109 systemic conditions such as hypertension or diabetes, those on systemic medications
110 including antihypertensives, analgesics, hormonal therapies, sedatives, or anti-seizure
111 medications, as well as individuals presenting with severe periodontal disease or
112 known allergies to probiotics.

113 The study utilized *L. reuteri* Prodentis lozenges, obtained from BioGaia
114 (Stockholm, Sweden). Each lozenge (800 mg) contained a minimum of 2 × 10^8 live
115 *Limosilactobacillus reuteri* Prodentis (previously classified as *Lactobacillus reuteri*).
116 This food supplement, specifically formulated for oral health, included a proprietary
117 combination of the *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289 strains, which
118 are recognized for their potential to support and maintain oral health.

119 To standardize oral hygiene practices, all participants were provided with a
120 standardized toothbrush and toothpaste for use throughout the study period.
121 Participants received oral hygiene instructions and were instructed to brush their teeth
122 twice daily. Each participant was administered one probiotic lozenge containing
123 *Limosilactobacillus reuteri* daily for 14 days, to be taken once per day after morning
124 toothbrushing and prior to breakfast. Plaque samples were collected from participants
125 on the day of enrolment, prior to the initiation of probiotic lozenge consumption, and
126 again on the 14th day following the completion of the probiotic regimen.

127 Before sample collection, mandatory rapid antigen test for SARS-CoV-2
128 detection had to be taken by the subjects in response to the COVID-19 pandemic (as
129 of 30 September 2020 when collecting the samples) and the test result had to be
130 negative for the subject partaking in the study. In brief, the participants were instructed
131 not to eat or drink anything two hours prior to collection of sample. Samples were
132 collected using sterile cotton buds and swabbed from
133 buccal/mesial/distal/lingual/occlusal surfaces of the index teeth of the subjects. The

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134 plaque samples were stored in sterile falcon tubes with 5 mL of phosphate-buffered
135 saline (PBS).

136 Furthermore, clinical data, including the Oral Hygiene Index-Simplified (OHI-S)
137 score and the Papilla Bleeding Index (PBI), were recorded at each visit. Overall, twenty
138 subjects had enrolled and fulfilled all requirements and inclusion criteria, as twenty
139 subjects were proved to be sufficient to provide valid data as a pilot study.[37,38] Plaque
140 samples from the subjects were continued unto the following downstream analysis.
141

142 **RNA Extraction, cDNA Synthesis and Quantification**

143 RNA from the sample was extracted using TRIzol reagent methodology as
144 instructed by the manufacturer (Thermo Fisher, Waltham, MA). The extracted RNA
145 was synthesized into cDNA using ReverTra Ace™ qPCR RT Master Mix with gDNA
146 Remover (Toyobo, Japan). The mixture for cDNA synthesis is described in Table 1.
147 The cDNA was then quantified using an Invitrogen™ Qubit 3.0 Fluorometer (Thermo
148 Fisher Scientific, Waltham, MA). The cDNA was stored at -20°C for storage of directly
149 used for downstream analysis.
150

151 **qPCR Analysis**

152 Amplification and detection by qPCR (Applied Biosystems, Waltham, MA) were
153 performed. The components of the qPCR Master Mix are listed in Table 2. Using a
154 specific kit, namely HOT FIREpol EvaGreen® qPCR Mix (Solis Biodyne, Tartu,
155 Estonia) which was activated by incubation at 95°C for 10 minutes. This was followed
156 by 40 cycles of denaturation at 95°C for 10 seconds, annealing temperature at
157 60−65°C (Table 3), and elongation at 72°C for 20 seconds. For templates longer than
158 150 bp, the annealing and elongation times were extended to 30 seconds. Actin gene
159 encoding as the housekeeping gene was used for normalization purposes. qPCR was
160 performed on cDNA. qPCR was performed using the primers listed in Table 3.
161

162 **Data Analysis and Outcome Analysis**

163 The data were analysed using the Shapiro−Wilk normality test ($p > 0.05$). For
164 data with a normal distribution, a paired *t*-test was applied. ($p < 0.05$). The software
165 used for the analysis is Statistical Package for the Social Sciences (SPSS) version 27
166 (IBM, Armonk, NY). The outcome analysis are the quantitative data presented from

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167 the RT-qPCR instrument. This data can then be correlated with previous study that
168 also conducted such research to ensure the validity of this data.
169

170 **Results:**

171 Based on the results of the qPCR test, the expression of *BCR1* (Graph 1A and
172 1B), *ACE2* (Graph 2A and 2B), *fadA* (Graph 3A and 3B), *aid1* (Graph 4A and 4B), *gelE*
173 (Graph 5A and 5B), and *atlA* (Graph 6A and 6B), on average, were decreased when
174 comparing the day-0 prior to probiotic consumption and day-14 after the subjects
175 consumed the probiotic *L. reuteri*.

176 As shown by the results of the Shapiro−Wilk normality test, all the data were
177 normally distributed ($p > 0.05$). The data were analysed using a paired *t*-test, with a
178 significance level of $p < 0.05$. The results of the paired *t*-test revealed a significant
179 difference in the comparison of the *BCR1* and *ACE2* gene expression data. As shown
180 in Table 4, there was a significant difference ($p < 0.05$) in the expression of *BCR1,*
181 *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes after consuming the probiotic *L. reuteri*.

182

183 **Discussion**:

184 The use of fixed orthodontic appliances often causes poor oral hygiene, thus
185 facilitating microorganism accumulation and various pathological conditions in the oral
186 cavity, such as fungal infections.[39] A previous study revealed an increase in *Candida*
187 in saliva after using fixed orthodontic appliances. The authors attributed this to the
188 design of fixed orthodontic appliances, which creates a space for the retention of food
189 waste.[40] Thus, patients must be instructed about good oral hygiene practices after
190 orthodontic treatment. [38] Fixed orthodontic appliances also induce changes in buffer
191 capacity, salivary flow rates, and acidity (pH), leading to plaque accumulation and an
192 increase in caries and periodontal disease.[41-43] Based on this information, we
193 attempted this study to prove the benefits of consuming probiotics lozenges as a
194 supplement for patients using fixed orthodontic appliances.

195 Biofilm formation is an important virulence factor of *F. nucleatum* due to its higher
196 resistance to host defence or antibacterial agents compared to planktonic cells.[44]
197 Several studies detected increased numbers of *Porphyromonas gingivalis, F.*
198 *nucleatum, P. intermedia,* and *Tannerella forsythia* after the use of fixed orthodontic
199 appliances.[15,45,46] They also reported that *F. nucleatum* increased the risk of
200 periodontitis in orthodontic patients due to a conducive environment for anaerobic
201 bacteria.[15,45,46] Biofilm formation is also a contributing factor to *E. faecalis* colonization
202 and infection. Biofilms develop through various processes by which bacteria adhere

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203 to surfaces, decompose complex matrices, and develop into bacterial colonies, which
204 adhere to surfaces.[47]

205 In this study, we used the qPCR method and $2^{-\Delta\Delta CT}$ formula to calculate target
206 gene expression. As shown by the results, the probiotic *L. reuteri* affected the
207 expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes and the formation of *C.*
208 *albicans, F. nucleatum*, and *E. faecalis* biofilms. Based on the Shapiro−Wilk normality
209 test, all the data were normally distributed, with $p > 0.05$. The paired *t*-test results
210 revealed significant differences in the expression *of BCR1, ACE2, fadA*, *aid1*, *gelE*,
211 and *atlA* genes (all $p < 0.05$).

212 Based on the results of this study, the probiotic *L. reuteri* significantly
213 downregulated the transcription of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes. In
214 gene expression, the process of translating genetic information in the form of
215 sequence of bases of DNA or RNA into proteins.[48] Through gene expression
216 measurement, it is possible to assess qualitatively and quantitatively the effect of a
217 treatment, such as the administration of a drug compound.[49] Gene expression in
218 microorganisms is involved in regulating cell-cell communication, carbohydrate
219 metabolism, adherence, and adaptation to the surrounding environment. A decrease
220 in the expression of specific genes can reduce microorganism colonization and
221 microorganism numbers.[50]

222 *BCR1*, a major gene transcription factor, produces an adhesin protein, which
223 facilitates *C. albicans* attachment to mucosal surfaces, which is a critical stage of
224 infection.[51] Deletion of *BCR1* eliminates *C. albicans* gene function, resulting in a
225 decrease in biofilm formation.[52] This was supported by the results of the present study,
226 which revealed a statistically significant decrease in *BCR1* gene expression after
227 consuming the probiotic.

228 The expression of *ACE2* also decreased based on the results of the statistical
229 tests. With deletion of *ACE2*, *C. albicans* is unable to form hyphal cells, and thus
230 biofilm formation is inhibited.[30] An earlier in vitro study showed that probiotics have
231 antifungal effects against *C. albicans* in the oral cavity. Regular use of probiotics
232 helped to inhibit *Candida* biofilms and reduced *Candida* colonization in the oral cavity,
233 thereby reducing the possibility of candidiasis infection.[53]

234 FadA protein is the main *F. nucleatum* virulence factor and mediates microbial
235 attachment and colonization.[8] Based on the results of this study, the probiotic *L. reuteri*

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236 appears to influence the pathogenicity of *F. nucleatum* adhesion molecules and
237 colonization and affect biofilm formation through decreased expression of the *fadA*
238 gene.[35] Various *F. nucleatum* adhesins mediate adhesion and aggregation and
239 function as coaggregation intermediaries in the formation and maturation of dental
240 biofilms.[54] The interaction of Fusobacterium with other species is largely mediated by
241 the adhesin genes *radD* and *aid1*.[55] The *aid1* gene plays a role in interspecies
242 interactions, colonization, and aggregation of *F. nucleatum*. In a previous study,
243 inactivation of the *aid1* gene decreased the ability of *F. nucleatum* to aggregate,
244 especially with *Streptococcus* spp. or *E. faecalis*.[14] As shown in earlier studies,
245 probiotics can affect the expression of genes involved in cell adhesion, quorum
246 sensing (QS), virulence factors, and biofilm formation.[56,57]

247 The *E. faecalis gelE* gene has the ability to hydrolyse gelatine, collagen, fibrin,
248 and other peptides.[58] Gene *gelE* is a virulence factor in infection formation through
249 bacterial attachment and biofilm formation.[22] In *E. faecalis*, biofilm formation is
250 regulated by QS, where Fsr regulates the expression of the *gelE* gene.[59] Fsr regulates
251 the formation of *E. faecalis* biofilms through its product *gelE* and serine proteases.[60]
252 QS is a molecular mechanism by which bacterial cells communicate with each other
253 via signalling molecules in biofilms. If the protease encoded by a signalling factor is
254 decreased, communication between bacteria and biofilm formation will be disrupted.[57]
255 GelE can also activate *atlA*, which is responsible for eDNA release at the biofilm
256 maturation stage.[61]

257 AtlA is involved in the hydrolysis of peptidoglycan, which plays an important role
258 in separating cells division after replication.[22] AtlA plays a role in the biofilm maturation
259 stage of *E. faecalis*, during which eDNA is released and contributes to biofilm
260 attachment and stability, biofilm defects in primary attachment, and decreased biofilm
261 production.[20,23] This study focused on the probiotic *L. reuteri*, which has ability to
262 secrete antimicrobial substances and compete with oral pathogens for adhesion to
263 mucosa. In addition, *L. reuteri* can adapt and change the pH of the surrounding
264 environment, thereby inhibiting the growth of oral pathogens.[62] The antimicrobial
265 substances secreted by *L. reuteri* are reuterin and reutericycline.[63] Reuterin and
266 reutericycline are broad-spectrum antimicrobial agents that are effective against gram-
267 positive and negative bacteria, fungi, and protozoa by inhibiting microbial DNA
268 synthesis.[35,41]

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269 Many dental and oral health care products used daily now include probiotics. The
270 use of probiotics is increasing due to their advantages over chemical agents, namely
271 reducing the risk of antibiotic resistance.[64] Probiotics work by modulating the immune
272 system, producing antimicrobial substances, and inhibiting certain pathogenic
273 organisms by interfering with adhesion, colonization, and biofilm formation. They
274 inhibit the growth of pathogens via the production of various substances, such as lactic
275 acid and acetic acid, which penetrate the bacterial cell membrane and lower the
276 cytoplasmic pH of pathogenic bacteria. Hydrogen peroxide and bacteriocin can
277 destroy the cell membrane of pathogenic bacteria and inhibit the synthesis of
278 pathogenic DNA.[32,33,35,65] Based on the results of this study, 2 weeks of daily
279 consumption of the probiotic *L. reuteri* affected the process of biofilm formation by
280 downregulating the expression of *BCR1*, *ACE2, fadA, aid1, gelE,* and *atlA g*enes,
281 which function as adherent regulators and regulators of hyphae formation in biofilm
282 formation. Many previous studies demonstrated that the addition of probiotics to dental
283 and oral health care products. Probiotic can reduced pathogenic microorganisms in
284 plaque samples from patients using fixed orthodontic appliances.[41,64,66–68] Therefore,
285 it can be stated that the probiotic *L. reuteri* has good ability as an additional treatment
286 for dental and oral health in patients using fixed orthodontic appliances.
287 The limitation of this study is the small sample size and the fact that the sampling
288 had to be done during COVID-19 pandemic. Although the sample size is small, the
289 result of this study can still provide concise and significant result. In the future, there
290 should be larger sample size for conducting this research so the result can more
291 accurately represents the actual population. Daily consumption of probiotic lozenges
292 duration can also be increased for more precise and accurate results. On the other
293 hand, consumption of probiotic lozenges research can also be conducted for other
294 aspect of oral health diseases to promote the functionality and health-inducing aspect
295 of probiotic lozenges, such as antiinflammation.
296

297 **Conclusion:**

298 Within the limitation of this study, it can be concluded that the probiotic *L. reuteri*
299 influences the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in biofilm
300 formation. By reducing the expression of those genes, the probiotic *L. reuteri* can

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reduce biofilm formation such as dental plaque in patients using fixed orthodontic appliances.

Clinical significance: Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression, hence, helping increasing oral health of consumer. The results of this study help clinicians provide probiotic lozenges for patients to promote and maintain their oral health.

List of abbreviations:

^{o}C	: Celcius degree
ACE2	: Angiotensin Converting Enzyme 2
aid1	: adherence inducing determinant gene 1
atlA	: autolysin
BCR1	: Biofilm and Cell wall Regulator 1
cDNA	: copy Deoxyribonucleic Acid
CFU/mL	: Colony forming unit per mililiter
DNA	: Deoxyribonucleic Acid
eDNA	: extracellular Deoxyribonucleic Acid
fadA	: fusobacterium adhesion A
gelE	: gelatinase
radD	: arginine-inhibitable adhesin
RT-qPCR	: Quantitative Polymerase Chain Reaction

326 **References:**

327 1. Sihotang BM, Panjaitan M, Sitanggang JM, Putri A, Harahap FR. Perbedaan oral
328 hygiene pemakaian pesawat ortodonti cekat dan pesawat ortodonti lepasan
329 pada pasien klinik spesialis RS Herna. Jurnal Prima Medika Sains. 2020 Mar
330 15;2(1):1–5.
331 2. Gkantidis N, Christou P, Topouzelis N. The orthodontic-periodontic
332 interrelationship in integrated treatment challenges: a systematic review. J Oral
333 Rehabil. 2010 May 1;37(5):377–90.
334 3. Oktaviani V, Santoso O. Perbedaan Indeks Higiene Oral dan pH Plak Kelompok
335 Pemakai dan Bukan Pemakai Pesawat Ortodonti Cekat. 2016;5(1):8.
336 4. Bowen WH, Burne RA, Wu H, Koo H. Oral Biofilms: Pathogens, Matrix and
337 Polymicrobial Interactions in Microenvironments. Trends Microbiol. 2018
338 Mar;26(3):229–42.
339 5. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence.
340 2011;2(5):435–44.
341 6. Verrusio C, Iorio-Siciliano V, Blasi A, Leuci S, Adamo D, Nicolò M. The effect of
342 orthodontic treatment on periodontal tissue inflammation: A systematic review.
343 Quintessence Int. 2018;49(1):69–77.
344 7. Perkowski K, Baltaza W, Conn DB, Marczyńska-Stolarek M, Chomicz L.
345 Examination of oral biofilm microbiota in patients using fixed orthodontic
346 appliances in order to prevent risk factors for health complications. Ann Agric
347 Environ Med. 2019 Jun 17;26(2):231–5.
348 8. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. Curr Opin
349 Microbiol. 2015 Feb;0:141–7.
350 9. Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS. Bacterial
351 coaggregation: an integral process in the development of multi-species biofilms.
352 Trends Microbiol. 2003 Feb;11(2):94–100.
353 10. Thurnheer T, Karygianni L, Flury M, Belibasakis GN. Fusobacterium Species and
354 Subspecies Differentially Affect the Composition and Architecture of Supra- and
355 Subgingival Biofilms Models. Front Microbiol. 2019 Jul 30;10:1716.
356 11. Chew J, Zilm PS, Fuss JM, Gully NJ. A proteomic investigation of Fusobacterium
357 nucleatum alkaline-induced biofilms. BMC Microbiology. 2012 Sep 3;12(1):189.
358 12. Kaplan CW, Lux R, Haake SK, Shi W. The Fusobacterium nucleatum Outer
359 Membrane Protein RadD Is an Arginine-Inhibitable Adhesin Required for Inter-
360 Species Adherence and the Structured Architecture of Multi-Species Biofilm. Mol
361 Microbiol. 2009 Jan;71(1):35–47.
362 13. Cochrane K, Robinson AV, Holt RA, Allen-Vercoe E. A survey of Fusobacterium
363 nucleatum genes modulated by host cell infection. Microb Genom. 2020
364 Feb;6(2).
365 14. Kaplan A, Kaplan CW, He X, McHardy I, Shi W, Lux R. Characterization of aid1,
366 a novel gene involved in Fusobacterium nucleatum interspecies interactions.
367 Microb Ecol. 2014 Aug;68(2):379–87.
368 15. Liu P, Liu Y, Wang J, Guo Y, Zhang Y, Xiao S. Detection of Fusobacterium
369 Nucleatum and fadA Adhesin Gene in Patients with Orthodontic Gingivitis and
370 Non-Orthodontic Periodontal Inflammation. PLOS ONE. 2014 Jan
371 9;9(1):e85280.

JAYPEE

372 16. Brennan CA, Garrett WS. Fusobacterium nucleatum - symbiont, opportunist and
373 oncobacterium. Nat Rev Microbiol. 2019 Mar;17(3):156–66.
374 17. Wu T, Cen L, Kaplan C, Zhou X, Lux R, Shi W, et al. Cellular Components
375 Mediating Coadherence of Candida albicans and Fusobacterium nucleatum. J
376 Dent Res. 2015 Oct;94(10):1432–8.
377 18. Wang Q-Q, Zhang C-F, Chu C-H, Zhu X-F. Prevalence of Enterococcus faecalis
378 in saliva and filled root canals of teeth associated with apical periodontitis. Int J
379 Oral Sci. 2012 Apr;4(1):19–23.
380 19. Najafi K, Gholizadeh P, Tanomand A, Ahangarzadeh Rezaee M, Mahmood S,
381 Asgharzadeh M, et al. Oral Cavity infection by Enterococcus Faecalis: virulence
382 factors and pathogenesis. Reviews in Medical Microbiology. 2019 Jan 2;30.
383 20. Ch'ng J-H, Chong KKL, Lam LN, Wong JJ, Kline KA. Biofilm-associated infection
384 by enterococci. Nat Rev Microbiol. 2019 Jan;17(2):82–94.
385 21. Anderson AC, Jonas D, Huber I, Karygianni L, Wölber J, Hellwig E, et al.
386 Enterococcus faecalis from Food, Clinical Specimens, and Oral Sites:
387 Prevalence of Virulence Factors in Association with Biofilm Formation. Front
388 Microbiol. 2016 Jan 11;6:1534.
389 22. Stinemetz EK, Gao P, Pinkston KL, Montealegre MC, Murray BE, Harvey BR.
390 Processing of the major autolysin of E. faecalis, AtlA, by the zinc-
391 metalloprotease, GelE, impacts AtlA septal localization and cell separation.
392 PLOS ONE. 2017 Oct 19;12(10):e0186706.
393 23. Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, et al. Contribution of
394 Autolysin and Sortase A during Enterococcus faecalis DNA-Dependent Biofilm
395 Development. Infect Immun. 2009 Sep;77(9):3626–38.
396 24. Paganelli FL, Willems RJL, Jansen P, Hendrickx A, Zhang X, Bonten MJM, et al.
397 Enterococcus faecium Biofilm Formation: Identification of Major Autolysin
398 AtlAEfm, Associated Acm Surface Localization, and AtlAEfm-Independent
399 Extracellular DNA Release. mBio. 2013 Apr 16;4(2):e00154-13.
400 25. Putri MH, Herijulianti E. Ilmu Pencegahan Penyakit Jaringan Keras dan Jaringan
401 Pendukung Gigi | OPAC Integrasi | Online Public Access Catalog | Universitas
402 Gadjah Mada. RGC; 2010.
403 26. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent
404 public health problem. Clin Microbiol Rev. 2007;20(1):133–63.
405 27. Garcia MC, Lee JT, Ramsook CB, Alsteens D, Dufrêne YF, Lipke PN. A Role for
406 Amyloid in Cell Aggregation and Biofilm Formation. PLOS ONE.
407 2011;6(3):e17632.
408 28. Nobile CJ, Mitchell AP. Regulation of Cell-Surface Genes and Biofilm Formation
409 by the C. albicans Transcription Factor Bcr1p. Current Biology. 2005 Jun
410 21;15(12):1150–5.
411 29. Mulhern SM, Logue ME, Butler G. Candida albicans Transcription Factor Ace2
412 Regulates Metabolism and Is Required for Filamentation in Hypoxic Conditions.
413 Eukaryot Cell. 2006 Dec;5(12):2001–13.
414 30. Saputo S, Kumar A, Krysan DJ. Efg1 Directly Regulates ACE2 Expression To
415 Mediate Cross Talk between the cAMP/PKA and RAM Pathways during Candida
416 albicans Morphogenesis. Eukaryot Cell. 2014 Sep;13(9):1169–80.

417 31. Widyarman AS, Bachtiar EW, Bachtiar BM, Seneviratne CJ. Inhibitory effect of probiotic lactobacilli against Streptococcus mutans and Porphyromonas gingivalis biofilms. Scientific Dental Journal. 2019 May 1;3(2):50.

420 32. Widyarman AS, Ramajayanti T, Theodorea CF. Indonesian Strain of Probiotic Reduces the Initial Biofilm Colonization. The Open Dentistry Journal [Internet]. 2020 Nov 13 [cited 2022 Feb 13];14(1). Available from: https://opendentistryjournal.com/VOLUME/14/PAGE/544/FULLTEXT/

424 33. Widyarman AS, Theodorea CF. Novel Indigenous Probiotic Lactobacillus reuteri Strain Produces Anti-biofilm Reuterin against Pathogenic Periodontal Bacteria. Eur J Dent [Internet]. 2021 Jul 24 [cited 2022 Feb 13]; Available from: http://www.thieme-connect.de/DOI/DOI?10.1055/s-0041-1731591

428 34. Mu Q, Tavella VJ, Luo XM. Role of Lactobacillus reuteri in Human Health and Diseases. Frontiers in Microbiology [Internet]. 2018 [cited 2022 Feb 13];9. Available from: https://www.frontiersin.org/article/10.3389/fmicb.2018.00757

431 35. Department of Microbiology, Faculty of Dentistry, Trisakti University, Jakarta, 11440 Indonesia, Sari Widyarman A, Kiky Elysia Lazaroni N, Undergraduate Program, Faculty of Dentistry, Trisakti University, Jakarta, 11440 Indonesia. Persistent Endodontics Pathogens Biofilm Inhibited by Lactobacillus reuteri Indonesian Strain Lactobacillus reuteri Indonesian. J Dent Indones [Internet]. 2019 Dec 31 [cited 2022 Feb 13];26(3). Available from: https://scholarhub.ui.ac.id/jdi/vol26/iss3/7/

438 36. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol. 2008;226(5):1065–73.

440 37. Julious SA (2005) Sample size of 12 per group rule of thumb for a pilot study. Pharm Stat 4:287–291. https://doi.org/10.1002/pst.185

442 38. Johanson GA, Brooks GP (2010) Initial scale development: sample size for pilot studies. Educ Psychol Meas 70:394–400. https://doi.org/10.1177/0013164409355692.

445 39. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. J Oral Microbiol [Internet]. 2011;3. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3087208/

448 40. Khanpayeh E, Jafari AA, Tabatabaei Z. Comparison of salivary Candida profile in patients with fixed and removable orthodontic appliances therapy. Iran J Microbiol. 2014 Aug;6(4):263–8.

451 41. Widyarman AS, Hartono V, Marjani LI, 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. Journal of International Dental and Medical Research. 2018 Jan 1;11(2):628–33.

456 42. Restrepo M, Bussaneli DG, Jeremias F, Cordeiro RCL, Raveli DB, Magalhães AC, et al. Control of White Spot Lesions with Use of Fluoride Varnish or Chlorhexidine Gel During Orthodontic Treatment A Randomized Clinical Trial. J Clin Pediatr Dent. 2016;40(4):274–80.

460 43. Lu H, Tang H, Zhou T, Kang N. Assessment of the periodontal health status in patients undergoing orthodontic treatment with fixed appliances and Invisalign system. Medicine (Baltimore). 2018 Mar 30;97(13):e0248.

JAYPEE

463 44. Fitriasari N, Wahyuni IS. POTENSI PROBIOTIK DALAM TATALAKSANA ORAL
464 CANDIDIASIS: Ulasan Sistematik. ODONTO : Dental Journal. 2021 Jul
465 12;8(1):34–44.
466 45. Guo L, Feng Y, Guo H-G, Liu B-W, Zhang Y. Consequences of orthodontic
467 treatment in malocclusion patients: clinical and microbial effects in adults and
468 children. BMC Oral Health. 2016 Oct 28;16:112.
469 46. Alqerban A. Efficacy of antimicrobial photodynamic and photobiomodulation
470 therapy against Treponema denticola, fusobacterium nucleatum and human beta
471 defensin-2 levels in patients with gingivitis undergoing fixed orthodontic
472 treatment: A clinic-laboratory study. Photodiagnosis and Photodynamic Therapy.
473 2020 Mar 1;29:101659.
474 47. Willett JLE, Ji MM, Dunny GM. Exploiting biofilm phenotypes for functional
475 characterization of hypothetical genes in Enterococcus faecalis. npj Biofilms
476 Microbiomes. 2019 Sep 19;5(1):1–14.
477 48. Sinaga E. Regulasi Ekspresi Gen. Regulasi Ekspresi Gen. 2011;1–24.
478 49. Van Peer G, Mestdagh P, Vandesompele J. Accurate RT-qPCR gene expression
479 analysis on cell culture lysates. Sci Rep. 2012 Jan 13;2:222.
480 50. Shemesh M, Tam A, Steinberg D 2007. Expression of biofilm-associated genes
481 of Streptococcus mutans in response to glucose and sucrose. Journal of Medical
482 Microbiology. 56(11):1528–35.
483 51. Nikoomanesh F, Department of medical mycology, Faculty of medical science,
484 Tarbiat Modares University, Tehran, Iran, Roudbarmohammadi S, Department
485 of medical mycology, Faculty of medical science, Tarbiat Modares University,
486 Tehran, Iran, Roudbary M, Department of Medical Mycology and Parasitology,
487 School of Medicine, Iran University of Medical Sciences, Tehran, Iran, et al.
488 Investigation of bcr1 Gene Expression in Candida albicans Isolates by RT-PCR
489 Technique and its Impact on Biofilm Formation. iem. 2016 Jan 1;2(1):22–4.
490 52. Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP. Divergent Targets
491 of Candida albicans Biofilm Regulator Bcr1 In Vitro and In Vivo. Eukaryotic Cell.
492 2012 Jul;11(7):896–904.
493 53. Matsubara VH, Bandara HMHN, Mayer MPA, Samaranayake LP. Probiotics as
494 Antifungals in Mucosal Candidiasis. Clin Infect Dis. 2016;62(9):1143–53.
495 54. Li Q, Tan L, Wang H, Kou Y, Shi X, Zhang S, et al. Fusobacterium nucleatum
496 Interaction with Pseudomonas aeruginosa Induces Biofilm-Associated Antibiotic
497 Tolerance via Fusobacterium Adhesin A. ACS Infect Dis. 2020 Jul 10;6(7):1686–
498 96.
499 55. Kaplan A. Identification and Characterization of a Novel Lipoprotein in
500 Fusobacterium nucleatum [Internet]. UCLA; 2015 [cited 2022 Feb 13]. Available
501 from: https://escholarship.org/uc/item/3r90n33c
502 56. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-
503 time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001
504 Dec;25(4):402–8.
505 57. Barzegari A, Kheyrolahzadeh K, Hosseiniyan Khatibi SM, Sharifi S, Memar MY,
506 Zununi Vahed S. The Battle of Probiotics and Their Derivatives Against Biofilms.
507 Infect Drug Resist. 2020 Feb 26;13:659–72.

JAYPEE

508 58. Guneser MB, Eldeniz AU. The effect of gelatinase production of Enterococcus faecalis on adhesion to dentin after irrigation with various endodontic irrigants. Acta Biomater Odontol Scand. 2016 Nov 15;2(1):144–9.

511 59. Hashem YA, Amin HM, Essam TM, Yassin AS, Aziz RK. Biofilm formation in enterococci: genotype-phenotype correlations and inhibition by vancomycin. Sci Rep. 2017 Jul 18;7(1):5733.

514 60. Wang L, Dong M, Zheng J, Song Q, Yin W, Li J, et al. Relationship of biofilm formation and gelE gene expression in Enterococcus faecalis recovered from root canals in patients requiring endodontic retreatment. J Endod. 2011 May;37(5):631–6.

518 61. Ali L, Goraya MU, Arafat Y, Ajmal M, Chen J-L, Yu D. Molecular Mechanism of Quorum-Sensing in Enterococcus faecalis: Its Role in Virulence and Therapeutic Approaches. Int J Mol Sci. 2017 May 3;18(5):960.

521 62. Bonifait L. Probiotics for Oral Health: Myth or Reality? 2009;75(8):6.

522 63. Urbańska M, Szajewska H. The efficacy of Lactobacillus reuteri DSM 17938 in infants and children: a review of the current evidence. Eur J Pediatr. 2014;173(10):1327–37.

525 64. Benic GZ, Farella M, Morgan XC, Viswam J, Heng NC, Cannon RD, et al. Oral probiotics reduce halitosis in patients wearing orthodontic braces: a randomized, triple-blind, placebo-controlled trial. J Breath Res. 2019 May 31;13(3):036010.

528 65. Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, Gómez-Llorente C, Gil A. Probiotic mechanisms of action. Ann Nutr Metab. 2012;61(2):160–74.

530 66. Widyarman AS, Yunita ST, Prasetyadi T. Consumption of Yogurt Containing Probiotic *Bifidobacterium lactis* Reduces *Streptococcus mutans* in Orthodontic Patients. Scientific Dental Journal. 2018 Jan 25;2(1):19–25.

533 67. Alp S, Baka ZM. Effects of probiotics on salivary *Streptecoccus mutans* and Lactobacillus levels in orthodontic patients. Am J Orthod Dentofacial Orthop. 2018 Oct;154(4):517–23.

536 68. Goyal N, Shamanna PU, Varughese ST, Abraham R, Antony B, Emmatty R, et al. Effects of amine fluoride and probiotic mouthwash on levels of *Porphyromonas gingivalis* in orthodontic patients: A randomized controlled trial. J Indian Soc Periodontol. 2019;23(4):339–44.

540 69. Ranjith K, Kalyana Chakravarthy S, Adicherla H, Sharma S, Shivaji S. Temporal Expression of Genes in Biofilm-Forming Ocular *Candida albicans* Isolated From Patients With Keratitis and Orbital Cellulitis. Investigative Ophthalmology & Visual Science. 2018 Jan 25;59(1):528–38.

544 70. Nailis H, Coenye T, Van Nieuwerburgh F, Deforce D, Nelis HJ. Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. BMC Mol Biol. 2006 Aug 4;7:25.

547 71. Vos M, Quince C, Pijl AS, Hollander M de, Kowalchuk GA. A Comparison of rpoB and 16S rRNA as Markers in Pyrosequencing Studies of Bacterial Diversity. PLOS ONE. 2012 Feb 15;7(2):e30600.

550 72. Zheng J-X, Wu Y, Lin Z-W, Pu Z-Y, Yao W-M, Chen Z, et al. Characteristics of and Virulence Factors Associated with Biofilm Formation in Clinical Enterococcus faecalis Isolates in China. Front Microbiol. 2017 Nov 24;8:2338

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556 ## Figures and figure legends

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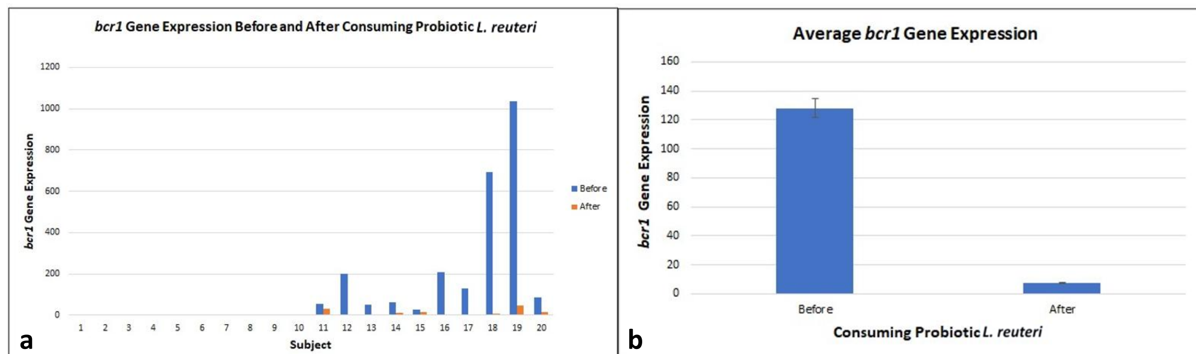

558

559 **Graph 1**. (**a**) A graph showing bcr1 gene expression in plaque samples *(N*= 20) as
560 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
561 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
562 *bcr1* gene expression in plaque samples as assessed by the RT-qPCR method and
563 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
564 lozenges.

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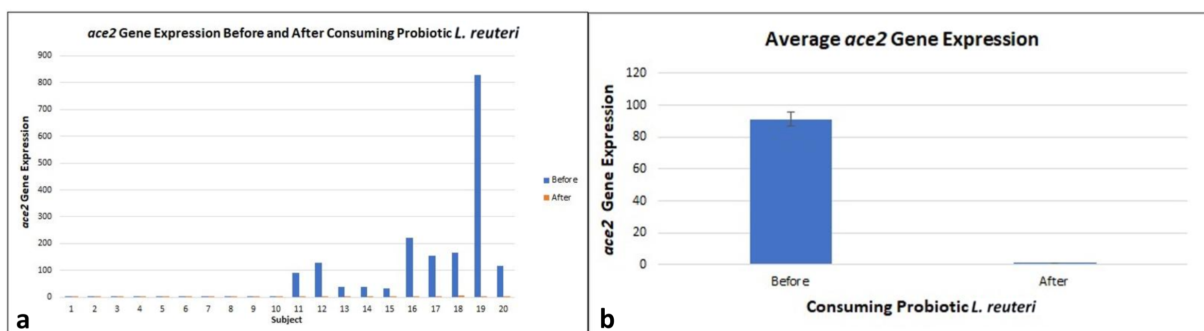

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568 **Graph 2**. (**a**) A graph showing ace2 gene expression in plaque samples *(N*= 20) as
569 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
570 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
571 *ace2* gene expression in plaque samples as assessed by the RT-qPCR method and
572 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
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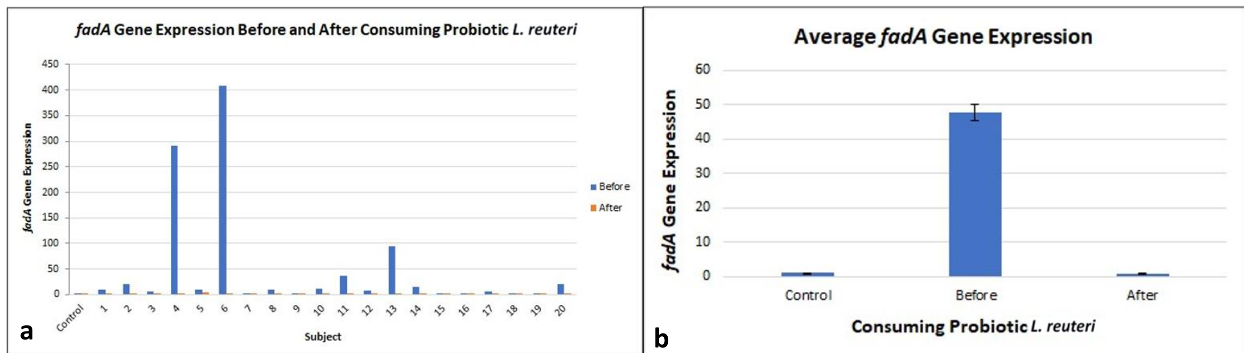

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580
581 **Graph 3**. (**a**) A graph showing fadA gene expression in plaque samples *(N*= 20) as
582 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
583 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
584 *fadA* gene expression in plaque samples as assessed by the RT-qPCR method and
585 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
586 lozenges.
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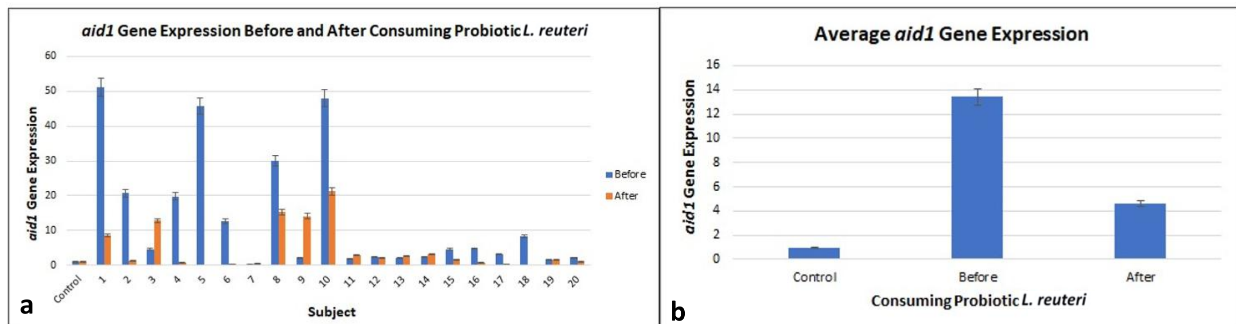

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591 **Graph 4**. (**a**) A graph showing aid1 gene expression in plaque samples *(N*= 20) as
592 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
593 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of
594 *aid1* gene expression in plaque samples as assessed by the RT-qPCR method and
595 the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic
596 lozenges.
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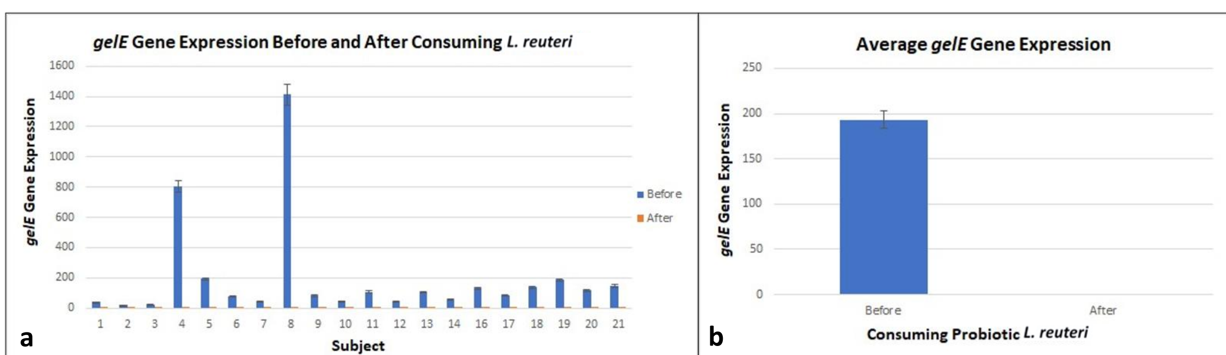

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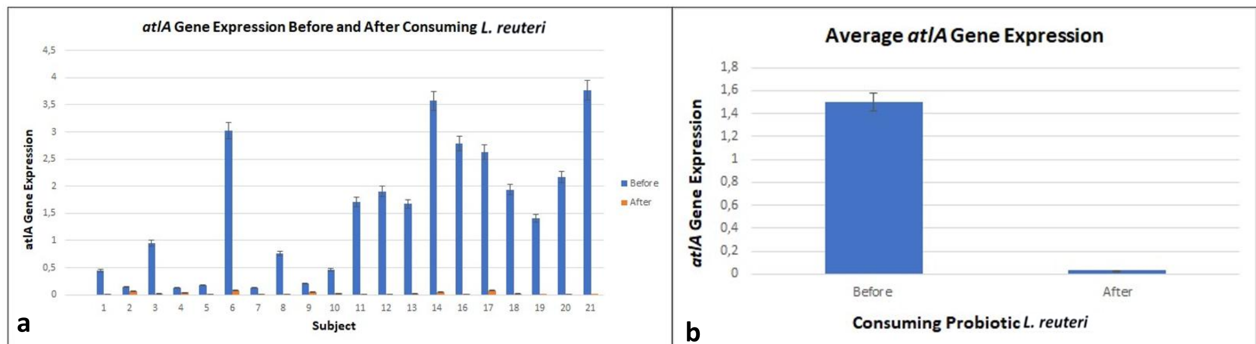
599 **Graph 5.** (**a**) A graph showing gelE gene expression in plaque samples *(N*= 20) as
600 assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before
601 and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of

gelE gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges.



Graph 6. (**a**) A graph showing atlA gene expression in plaque samples (*N*= 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges (**b**) A graph showing the average of *atlA* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges.

621 ## Tables and table legends

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623 **Table 1.** Reagent components for DNase I Reaction Solution

Component	Volume
4x DN *Master Mix*	2 mL
RNA *template*	0,5 pg – 0,5 mg
Nuclease-free Water	X mL
Total Volume	**8 mL**

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625 **Table 2.** Components of Master Mix RT-qPCR

Component	Volume
5 x HOT FIREPol EvaGreen® qPCR Mix Plus	4 mL
Primer *Forward*	1 mL
Primer *Reverse*	1 mL
DNA *template*	2 mL
NFW	12 mL
Total	**20 mL**

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629 **Table 3.** *Primer sequence* for RT-qPCR

Genes	***Primer sequence***	
BCR1[51]	*forward*: 5'- CTTCAGCAGCTTCATTAACACCTA -3' *reverse*: 5'- TCTTGGATCAGGTGTACTTTTCAA- 3'	Initial denaturation of 95°C for 5 minutes; 40 Cycles of denaturation at 95°C for 1 minute and annealing at 58°C for 1 minute.
ACE2[69]	*forward* 5'- AGAATTGACCGTTGTCCGTGTAA G-3' *reverse*: 5'- AATGGGTGAATAAATCCCTCCCTA A-3'	Initial denaturation 95⁰C for 2 minutes; 40 Cycles of denaturation at 95°C for 30 seconds and annealing at 60°C for 1 minute.
Housekeeping gene C. albicans : *ACT1*[70]	*forward*: 5'- TTTCATCTTCTGTATCAGAGGAAC TTATTT-3' *reverse*: 5'- ATGGGATGAATCATCAAACAAGA G-3'	Initial denaturation 95°C for 10 minutes; 40 Cycles of denaturing 95°C for 15 seconds and annealing 60°C for 1 minute

fadA[15]	*forward*: 5' -CAC AAG CTG ACG CTG CTA GA- 3' *reverse*: 5' -TTA CCA GCT CTT AAA GCT TG- 3'	Initial incubation for 4 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55.8°C for 30 seconds, and elongation at 72°C for 40 seconds and the final elongation for 6 min.[14]
aid1[14]	*forward:* 5' -TACAGGAG GTGCCGTAGCAG-3' *reverse:* 5' -TTTTTGTTAATTCT CCAGCTCCA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.[13]
Housekeeping gene *F. nucleatum*: *rpoB*[71]	*forward:* 5'-GGYTWYGAAGTNCGHGACGTDCA- 3' *reverse:* 5'-TGACGYTGCATGTTBGMR CCCATMA- 3'	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.

gelE[60]	*forward:* 5'- CGGAACATACTGCCGGTTTAGA - 3' *reverse:* 5'- TGGATTAGATGCACCCGAAAT - 3'	Initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 5 seconds, and annealing at 60°C for 30 seconds.
atlA[72]	*forward:* 5'- AATAATCAATCAGGAACGAATACG - 3' *reverse:* 5'- GCCACACTAACACCGAAT -3'	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.
Housekeeping gene E. faecalis: rpoA[72]	*forward:* 5'- GTGAAACCTGGTCGTGGCTA - 3' *reverse:* 5'- CGACGAACGGGTGTGTAGAT- 3'	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.

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7. Bukti konfirmasi artikel accepted

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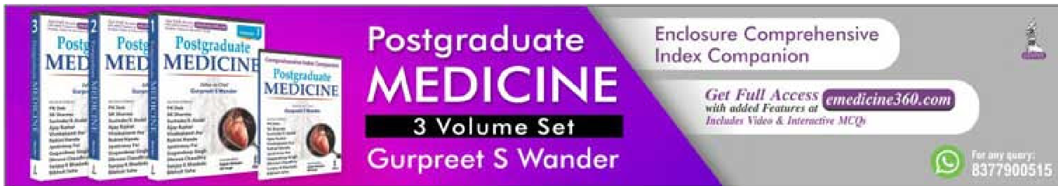

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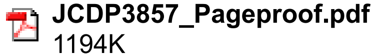

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ORIGINAL RESEARCH

Lactobacillus Reuteri Probiotic Consumption Reduced Various Virulence Gene Expression in Dental Plaque of Fixed Orthodontic Subjects

Q1 Joko Kusnoto[1], Siti S Safirah[2], Litayana RA Sitorus[3], Winnie Valentini[4], Armelia S Widyarman[5]

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Abstract

Aims: The aim of this study was to determine the effect of consuming lozenges containing *Lactobacillus reuteri* probiotic Prodentis lozenges on the expression of *BCR1, ACE2*, *fadA, aid1*, *gelE*, and *atlA* genes in dental biofilms of subjects using fixed orthodontic appliances.

Materials and methods: Plaque samples ($n = 20$) obtained from a previous study were used in this research. Each subject consumed *L. reuteri* probiotic lozenges (2×10^8 CFU/mL) each day for 2 weeks. RNA was extracted from the samples and synthesized into cDNA. The expression of the gene transcription factors *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes in biofilms of subjects who used fixed orthodontic appliances was detected using real time quantitative polymerase chain reaction (RT-qPCR).

Results: The expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes were decreased after consuming the *L. reuteri* probiotic lozenges for 2 weeks ($p < 0.05$).

Conclusion: Consuming *L. reuteri* probiotic lozenges would affect the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* in plaque from patients using fixed orthodontic appliances. By reducing the expression of the virulence genes, bacterial number would be reduced and biofilm production can also be reduced.

Clinical significance: Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression. Routine consumption of probiotic lozenges can help reduce potential bacterial infection and increase the oral health of patients using fixed orthodontic appliances.

Keywords: Biofilm, Gene expression, *Lactobacillus reuteri*, Orthodontic, Probiotic.

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Introduction

Orthodontic treatment is common today in the community. Among adults and children, orthodontic treatment may be undertaken for dental care or esthetic reasons.[1] Orthodontic treatment using fixed appliances aims to ensure proper occlusion and esthetic function, with appropriate tooth movement. Fixed orthodontic treatment can lead to changes in the oral environment and oral flora composition and an increase in the amount of plaque due to difficulty in maintaining oral hygiene.[2–5] In addition, excess composite around the base of the bracket that used in orthodontic treatment is an important factor that can cause plaque accumulation due to the presence of rough surfaces and cracks on the enamel composite surfaces.[3–5] Biofilm accumulation on teeth and soft tissues in the oral cavity can lead to caries, gingivitis, and periodontitis.[6,7]

Fusobacterium nucleatum is a dominant bacterial species that plays an important role in the formation of dental biofilms and periodontal tissue disease. In the formation of biofilms, *F. nucleatum* being a "bridging" or "linking" organism between initial bacterial colonization and final bacterial colonization, which are unable to bind to each other directly. *F. nucleatum* can also co-aggregate with various microbial species in the oral cavity.[8–10] *F. nucleatum* encodes several adhesion genes involved in interspecies interactions, including fusobacterium adhesion A (*fadA*), fusobacterial outer membrane protein A (*fomA*), *radD* (an arginine-inhibitable adhesin), and adherence inducing determinant gene 1 (*aid1*).[8,11–14] Fusobacterium adhesin A (FadA) is known to be involved in *F. nucleatum* invasion and adhesion to host cells and is highly conserved among oral Fusobacterium species.[8,15,16] FadA has been identified has a major virulence factor in *F. nucleatum* in interspecies interactions with *Streptococcus* mediated by *radD,* as it increases the binding specificity of *F. nucleatum* to other microbial species.[14] The arginine-inhibitable adhesion *radD* is required by *F. nucleatum* for co-adherence with various species of Gram-positive bacteria, such as streptococci (early colonizers), and fungal species, such as *Candida*.[11,12,17]

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Enterococcus faecalis (*E. faecalis*) is associated with chronic periodontitis and chronic apical periodontitis in failed root canal treatment.[18] *E. faecalis* is a Gram-positive aerobic bacterium. The severity of *E. faecalis* infection depends on the immune response and virulence factors, which can exacerbate infection and play a role in increasing biofilm formation.[19] There are several genes associated with *E. faecalis* biofilm formation, including gelatinase (*gelE*) and autolysin (*atlA*).[20] GelE in *E. faecalis* plaque or saliva isolates showed resistance to antibiotics and high biofilm formation ability.[21] atlA is the main peptidoglycan hydrolase or autolysin of *E. faecalis*.[22] AtlA plays a role in the biofilm maturation stage during which extracellular DNA (eDNA) is released and contributes to biofilm attachment and stability.[23,24]

An increase in the number of colonies of microorganisms also increased, one of which was *Candida albicans*, which causes infections of oral mucosa.[25,26] *C. albicans* has a protein in the form of an adhesive that mediates other microorganisms to adhere to abiotic and host surfaces to form biofilms.[27] Several *C. albicans* gene transcription factors, including biofilm and cell wall regulator 1 (*BCR1*) and angiotensin converting enzyme 2 (*ACE2*), play a role in the formation of biofilms. BCR1 acts as a major regulator of *C. albicans* biofilm formation.[28] The ACE2 transcription factor plays a role in fungal adherence, biofilm formation, and hyphal morphogenesis. In addition, ACE2 plays a role in regulating the expression of genes involved in cell wall separation and metabolism.[29] As shown in previous research, ACE2 is required for filamentation, and it can increase the number of pseudohyphae cells at the time of biofilm formation.[30]

Biofilm formation plays a role in increasing antibiotic resistance in bacterial cells. Therefore, an effective therapy is needed to prevent biofilm formation. The use of probiotics has been suggested as a promising approach to prevent and treat microbial diseases and biofilm activity in the oral cavity.[31,32] Several studies have proven that the use of probiotics has oral cavity health benefits, such as preventing caries and periodontal disease.[32,33] One commercial probiotic proven to be beneficial for oral health is *Lactobacillus reuteri*. The antimicrobial activity of *L. reuteri* inhibits colonization by pathogenic microbes and interacts the inhibition directly with host cells.[34] *L. reuteri* also inhibits the growth of *C. albicans* and *E. faecalis* biofilms.[35,36] However, no studies have investigated the effect of the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in dental plaque biofilms found in patient's oral environment with fixed orthodontic appliances. By analyzing the virulence genes found in several pathogenic bacteria, we can hypothesize that the downregulation of these genes will lead to less biofilm production and healthier oral health conditions during the duration of fixed orthodontic appliances usage. Thus, to bridge the knowledge gap, the aim of this study was to determine the effect of consuming lozenges containing the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes in biofilms from subjects using fixed orthodontic appliances.

Materials and Methods

Sample Collection

Subjects undergoing fixed orthodontic therapy were enrolled in this open-label prospective, clinical trial. Ethical approval for the study was granted by the Institutional Review Board of the Faculty of Dentistry, Universitas Indonesia (approval number: 100,701,020). The inclusion criteria specified participants aged 18 years or older who had been undergoing orthodontic treatment with fixed appliances for a minimum duration of 1 year and had not consumed probiotics or antibiotics in the preceding 3 months. The fixed orthodontic appliances used by the subjects are conventional metal braces fixed appliance. Exclusion criteria encompassed individuals with systemic conditions such as hypertension or diabetes, those on systemic medications including antihypertensives, analgesics, hormonal therapies, sedatives, or anti-seizure medications, as well as individuals presenting with severe periodontal disease or known allergies to probiotics.

The study utilized *L. reuteri* Prodentis lozenges, obtained from BioGaia (Stockholm, Sweden). Each lozenge (800 mg) contained a minimum of 2×10^8 live *Limosilactobacillus reuteri* Prodentis (previously classified as *L. reuteri*). This food supplement, specifically formulated for oral health, included a proprietary combination of the *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289 strains, which are recognized for their potential to support and maintain oral health.

To standardize oral hygiene practices, all participants were provided with a standardized toothbrush and toothpaste for use throughout the study period. Participants received oral hygiene instructions and were instructed to brush their teeth twice daily. Each participant was administered one probiotic lozenge containing *Limosilactobacillus reuteri* daily for 14 days, to be taken once per day after morning toothbrushing and prior to breakfast. Plaque samples were collected from participants on the day of enrolment, prior to the initiation of probiotic lozenge consumption, and again on the 14th day following the completion of the probiotic regimen.

Before sample collection, mandatory rapid antigen test for SARS-CoV-2 detection had to be taken by the subjects in response to the COVID-19 pandemic (as of 30 September 2020 when collecting the samples) and the test result had to be negative for the subject partaking in the study. In brief, the participants were instructed not to eat or drink anything 2 hours prior to collection of sample. Samples were collected using sterile cotton buds and swabbed from buccal/mesial/distal/lingual/occlusal surfaces of the index teeth of the subjects. The plaque samples were stored in sterile falcon tubes with 5 mL of phosphate-buffered saline (PBS).

Furthermore, clinical data, including the Oral Hygiene Index-Simplified score and the Papilla Bleeding Index, were recorded at each visit. Overall, 20 subjects had enrolled and fulfilled all requirements and inclusion criteria, as 20 subjects were proved to be sufficient to provide valid data as a pilot study.[37,38] Plaque samples from the subjects were continued unto the following downstream analysis.

RNA Extraction, cDNA Synthesis, and Quantification

RNA from the sample was extracted using TRIzol reagent methodology as instructed by the manufacturer (Thermo Fisher, Waltham, MA). The extracted RNA was synthesized into cDNA using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The mixture for cDNA synthesis is described in Table 1. The cDNA was then quantified using an Invitrogen™ Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). The cDNA was stored at –20°C for storage of directly used for downstream analysis.

qPCR Analysis

Amplification and detection by qPCR (Applied Biosystems, Waltham, MA) were performed. The components of the qPCR Master Mix are listed in Table 2. Using a specific kit, namely HOT FIREpol EvaGreen® qPCR Mix (Solis Biodyne, Tartu, Estonia) which was activated by

Table 1: Reagent components for DNase I reaction solution

Component	*Volume*
4 × DN Master Mix	2 mL
RNA template	0.5 pg – 0.5 mg
Nuclease-free water	X mL
Total volume	8 mL

Table 2: Components of Master Mix RT-qPCR

Component	*Volume*
5 × HOT FIREPol EvaGreen® qPCR Mix Plus	4 mL
Primer *Forward*	1 mL
Primer *Reverse*	1 mL
DNA *template*	2 mL
NFW	12 mL
Total	20 mL

incubation at 95°C for 10 minutes. This was followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing temperature at 60–65°C (Table 3), and elongation at 72°C for 20 seconds. For templates longer than 150 bp, the annealing and elongation times were extended to 30 seconds. Actin gene encoding as the housekeeping gene was used for normalization purposes. qPCR was performed on cDNA. qPCR was performed using the primers listed in Table 3.

Data Analysis and Outcome Analysis

The data were analyzed using the Shapiro–Wilk normality test ($p > 0.05$). For data with a normal distribution, a paired *t*-test was applied. ($p < 0.05$). The software used for the analysis is Statistical Package for the Social Sciences (SPSS) version 27 (IBM, Armonk, NY). The outcome analysis are the quantitative data presented from the RT-qPCR instrument. This data can then be correlated with previous study that also conducted such research to ensure the validity of this data.

Table 3: *Primer sequence* for RT-qPCR

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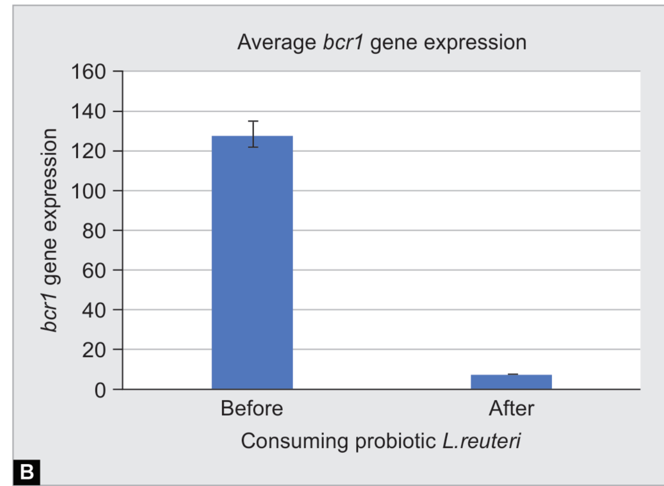
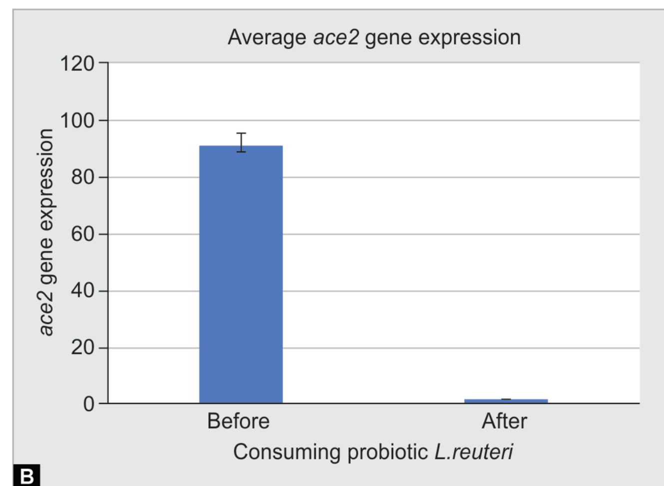
Genes	*Primer sequence*	
BCR1[39]	*forward*: 5′-CTTCAGCAGCTTCATTAACACCTA-3′ *reverse*: 5′-TCTTGGATCAGGTGTACTTTTCAA-3′	Initial denaturation of 95°C for 5 minutes; 40 cycles of denaturation at 95°C for 1 minute and annealing at 58°C for 1 minute.
ACE2[40]	*forward* 5′-AGAATTGACCGTTGTCCGTGTAAG-3′ *reverse*: 5′-AATGGGTGAATAAATCCCTCCCTAA-3′	Initial denaturation 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 30 seconds and annealing at 60°C for 1 minute.
Housekeeping gene C. albicans: *ACT1*[41]	*forward*: 5′-TTTCATCTTCTGTATCAGAGGAACTTATTT-3′ *reverse*: 5′-ATGGGATGAATCATCAAACAAGAG-3′	Initial denaturation 95°C for 10 minutes; 40 cycles of denaturing 95°C for 15 seconds and annealing 60°C for 1 minute.
fadA[15]	*forward*: 5′-CAC AAG CTG ACG CTG CTA GA- 3′ *reverse*: 5′-TTA CCA GCT CTT AAA GCT TG-3′	Initial incubation for 4 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55.8°C for 30 seconds, and elongation at 72°C for 40 seconds and the final elongation for 6 minutes.[14]
aid1[14]	*forward:* 5′-TACAGGAG GTGCCGTAGCAG-3′ *reverse:* 5′-TTTTTGTTAATTCT CCAGCTCCA-3′	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.[13]
Housekeeping gene *F. nucleatum*: *rpoB*[42]	*forward:* 5′-GGYTWYGAAGTNCGHGACGTDCA-3′ *reverse:* 5′-TGACGYTGCATGTTBGMR CCCATMA-3′	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.
gelE[43]	*forward:* 5′-CGGAACATACTGCCGGTTTAGA-3′ *reverse:* 5′-TGGATTAGATGCACCCGAAAT -3′	Initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 5 seconds, and annealing at 60°C for 30 seconds.
atlA[44]	*forward:* 5′-AATAATCAATCAGGAACGAATACG-3′ *reverse:* 5′-GCCACACTAACACCGAAT-3′	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.
Housekeeping gene E. faecalis: rpoA[44]	*forward:* 5′-GTGAAACCTGGTCGTGGCTA-3′ *reverse:* 5′-CGACGAACGGGTGTGTAGAT-3′	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.



Figs 1A and B: (A) A graph showing *bcr1* gene expression in plaque samples (*N* = 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *bcr1* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges



Figs 2A and B: (A) A graph showing *ACE2* gene expression in plaque samples (*N* = 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *ACE2* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Results

Based on the results of the qPCR test, the expression of *BCR1* (Fig. 1), *ACE2* (Fig. 2), *fadA* (Fig. 3), *aid1* (Fig. 4), *gelE* (Fig. 5), and *atlA* (Fig. 6), on average, were decreased when comparing the day-0 prior to probiotic consumption and day-14 after the subjects consumed the probiotic *L. reuteri*.

As shown by the results of the Shapiro–Wilk normality test, all the data were normally distributed ($p > 0.05$). The data were analyzed using a paired *t*-test, with a significance level of $p < 0.05$. The results of the paired *t*-test revealed a significant difference in the comparison of the *BCR1* and *ACE2* gene expression data. There was a significant difference ($p < 0.05$) in the expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes after consuming the probiotic *L. reuteri*.

Discussion

The use of fixed orthodontic appliances often causes poor oral hygiene, thus facilitating microorganism accumulation and various pathological conditions in the oral cavity, such as fungal infections.[45] A previous study revealed an increase in *Candida* in saliva after using fixed orthodontic appliances. The authors attributed this to the design of fixed orthodontic appliances, which creates a space for the retention of food waste.[46] Thus, patients must be instructed about good oral hygiene practices after orthodontic treatment.[38] Fixed orthodontic appliances also induce changes in buffer capacity, salivary flow rates, and acidity (pH), leading to plaque accumulation and an increase in caries and periodontal disease.[47–49] Based on this information, we attempted this study to prove the benefits of consuming probiotics lozenges as a supplement for patients using fixed orthodontic appliances.

Biofilm formation is an important virulence factor of *F. nucleatum* due to its higher resistance to host defence or antibacterial agents compared to planktonic cells.[50] Several studies detected increased numbers of *Porphyromonas gingivalis, F. nucleatum, P. intermedia,* and *Tannerella forsythia* after the use of fixed orthodontic appliances.[15,51,52] They also reported that *F. nucleatum* increased

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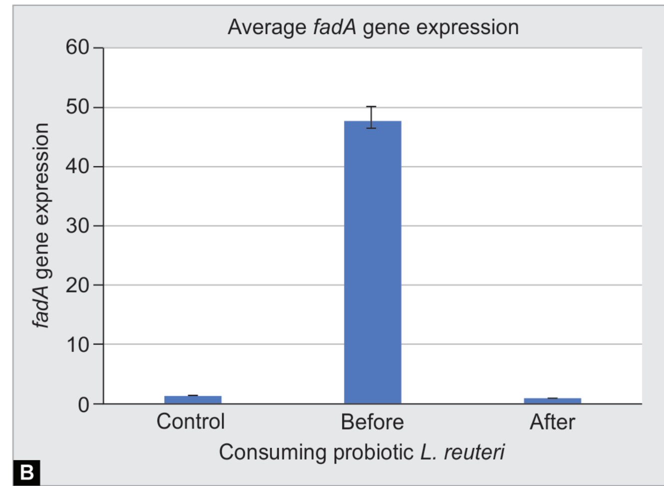
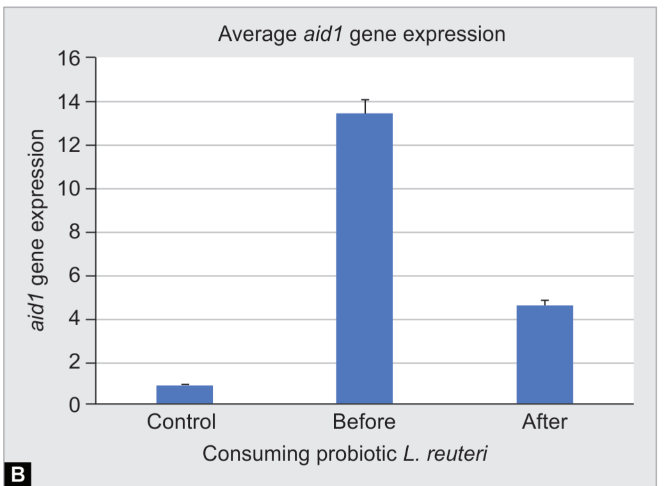
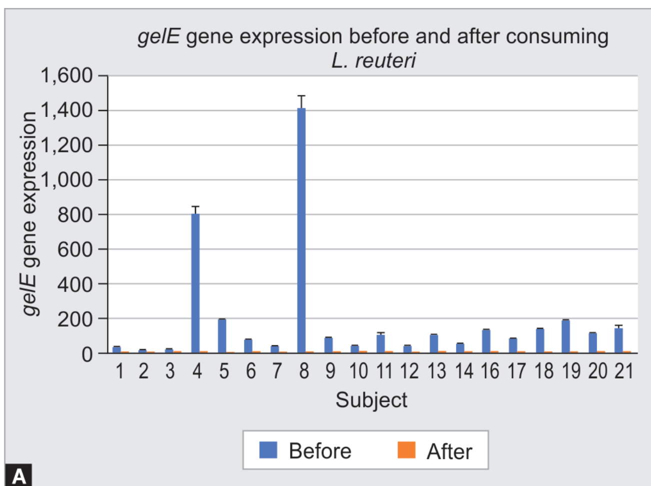


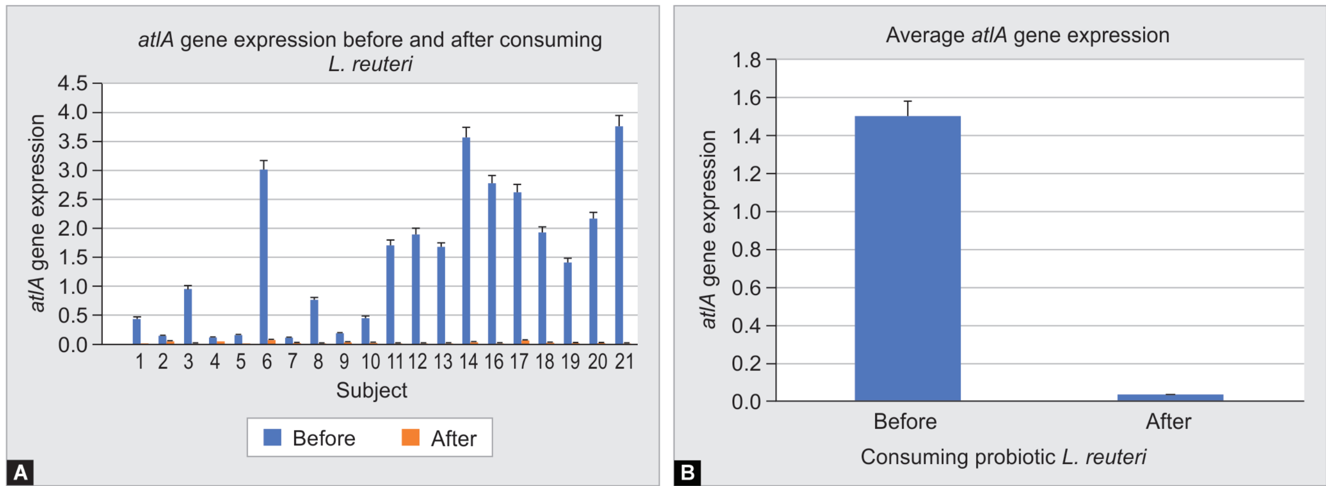
Figs 3A and B: (A) A graph showing *fadA* gene expression in plaque samples (*N* = 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *fadA* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges



Figs 4A and B: (A) A graph showing *aid1* gene expression in plaque samples (*N* = 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *aid1* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges



Figs 5A and B: (A) A graph showing *gelE* gene expression in plaque samples (*N* = 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *gelE* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges



Figs 6A and B: (A) A graph showing *atlA* gene expression in plaque samples *(N* = 20) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *atlA* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

the risk of periodontitis in orthodontic patients due to a conducive environment for anaerobic bacteria.[15,51,52] Biofilm formation is also a contributing factor to *E. faecalis* colonization and infection. Biofilms develop through various processes by which bacteria adhere to surfaces, decompose complex matrices, and develop into bacterial colonies, which adhere to surfaces.[53]

In this study, we used the qPCR method and $2^{-\Delta\Delta CT}$ formula to calculate target gene expression. As shown by the results, the probiotic *L. reuteri* affected the expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes and the formation of *C. albicans, F. nucleatum*, and *E. faecalis* biofilms. Based on the Shapiro–Wilk normality test, all the data were normally distributed, with $p > 0.05$. The paired *t*-test results revealed significant differences in the expression *of BCR1, ACE2, fadA*, *aid1*, *gelE*, and *atlA* genes (all $p < 0.05$).

Based on the results of this study, the probiotic *L. reuteri* significantly downregulated the transcription of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes. In gene expression, the process of translating genetic information in the form of sequence of bases of DNA or RNA into proteins.[54] Through gene expression measurement, it is possible to assess qualitatively and quantitatively the effect of a treatment, such as the administration of a drug compound.[55] Gene expression in microorganisms is involved in regulating cell-cell communication, carbohydrate metabolism, adherence, and adaptation to the surrounding environment. A decrease in the expression of specific genes can reduce microorganism colonization and microorganism numbers.[56]

BCR1, a major gene transcription factor, produces an adhesin protein, which facilitates *C. albicans* attachment to mucosal surfaces, which is a critical stage of infection.[39] Deletion of *BCR1* eliminates *C. albicans* gene function, resulting in a decrease in biofilm formation.[57] This was supported by the results of the present study, which revealed a statistically significant decrease in *BCR1* gene expression after consuming the probiotic.

The expression of *ACE2* also decreased based on the results of the statistical tests. With deletion of *ACE2*, *C. albicans* is unable to form hyphal cells, and thus biofilm formation is inhibited.[30] An earlier in vitro study showed that probiotics have antifungal effects against *C. albicans* in the oral cavity. Regular use of probiotics helped to inhibit *Candida* biofilms and reduced *Candida* colonization in the oral cavity, thereby reducing the possibility of candidiasis infection.[58]

FadA protein is the main *F. nucleatum* virulence factor and mediates microbial attachment and colonization.[8] Based on the results of this study, the probiotic *L. reuteri* appears to influence the pathogenicity of *F. nucleatum* adhesion molecules and colonization and affect biofilm formation through decreased expression of the *fadA* gene.[35] Various *F. nucleatum* adhesins mediate adhesion and aggregation and function as coaggregation intermediaries in the formation and maturation of dental biofilms.[59] The interaction of Fusobacterium with other species is largely mediated by the adhesin genes *radD* and *aid1*.[60] The *aid1* gene plays a role in interspecies interactions, colonization, and aggregation of *F. nucleatum*. In a previous study, inactivation of the *aid1* gene decreased the ability of *F. nucleatum* to aggregate, especially with *Streptococcus* spp. or *E. faecalis*.[14] As shown in earlier studies, probiotics can affect the expression of genes involved in cell adhesion, quorum sensing (QS), virulence factors, and biofilm formation.[61,62]

The *E. faecalis gelE* gene has the ability to hydrolyse gelatine, collagen, fibrin, and other peptides.[63] Gene *gelE* is a virulence factor in infection formation through bacterial attachment and biofilm formation.[22] In *E. faecalis*, biofilm formation is regulated by QS, where Fsr regulates the expression of the *gelE* gene.[64] Fsr regulates the formation of *E. faecalis* biofilms through its product *gelE* and serine proteases.[43] QS is a molecular mechanism by which bacterial cells communicate with each other via signalling molecules in biofilms. If the protease encoded by a signalling factor is decreased, communication between bacteria and biofilm formation will be disrupted.[62] GelE can also activate *atlA*, which is responsible for eDNA release at the biofilm maturation stage.[65]

AtlA is involved in the hydrolysis of peptidoglycan, which plays an important role in separating cells division after replication.[22] AtlA plays a role in the biofilm maturation stage of *E. faecalis*, during which eDNA is released and contributes to biofilm attachment and stability, biofilm defects in primary attachment, and decreased biofilm production.[20,23] This study focused on the probiotic *L. reuteri*, which has ability to secrete antimicrobial substances and compete with oral pathogens for adhesion to mucosa. In addition, *L. reuteri* can adapt and change the pH of the surrounding

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environment, thereby inhibiting the growth of oral pathogens.[66] The antimicrobial substances secreted by *L. reuteri* are reuterin and reutericycline.[67] Reuterin and reutericycline are broad-spectrum antimicrobial agents that are effective against Gram-positive and -negative bacteria, fungi, and protozoa by inhibiting microbial DNA synthesis.[35,47]

Many dental and oral health care products used daily now include probiotics. The use of probiotics is increasing due to their advantages over chemical agents, namely reducing the risk of antibiotic resistance.[68] Probiotics work by modulating the immune system, producing antimicrobial substances, and inhibiting certain pathogenic organisms by interfering with adhesion, colonization, and biofilm formation. They inhibit the growth of pathogens via the production of various substances, such as lactic acid and acetic acid, which penetrate the bacterial cell membrane and lower the cytoplasmic pH of pathogenic bacteria. Hydrogen peroxide and bacteriocin can destroy the cell membrane of pathogenic bacteria and inhibit the synthesis of pathogenic DNA.[32,33,35,69] Based on the results of this study, 2 weeks of daily consumption of the probiotic *L. reuteri* affected the process of biofilm formation by downregulating the expression of *BCR1*, *ACE2, fadA, aid1, gelE,* and *atlA g*enes, which function as adherent regulators and regulators of hyphae formation in biofilm formation. Many previous studies demonstrated that the addition of probiotics to dental and oral health care products. Probiotic can reduce pathogenic microorganisms in plaque samples from patients using fixed orthodontic appliances.[47,68,70–72] Therefore, it can be stated that the probiotic *L. reuteri* has good ability as an additional treatment for dental and oral health in patients using fixed orthodontic appliances.

The limitation of this study is the small sample size and the fact that the sampling had to be done during COVID-19 pandemic. Although the sample size is small, the result of this study can still provide concise and significant result. In the future, there should be larger sample size for conducting this research so the results can more accurately represent the actual population. Daily consumption of probiotic lozenges duration can also be increased for more precise and accurate results. On the other hand, consumption of probiotic lozenges research can also be conducted for other aspect of oral health diseases to promote the functionality and health-inducing aspect of probiotic lozenges, such as antiinflammation.

Conclusion

Within the limitation of this study, it can be concluded that the probiotic *L. reuteri* influences the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in biofilm formation. By reducing the expression of those genes, the probiotic *L. reuteri* can reduce biofilm formation, such as dental plaque, in patients using fixed orthodontic appliances.

Clinical Significance

Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression, hence, helping increasing oral health of consumer. The results of this study help clinicians provide probiotic lozenges for patients to promote and maintain their oral health.

References

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1. Sihotang BM, Panjaitan M, et al. Perbedaan oral hygiene pemakaian pesawat ortodonti cekat dan pesawat ortodonti lepasan pada pasien klinik spesialis RS Herna. J Prima Medika Sains 202015;2(1):1–5.
2. Gkantidis N, Christou P, Topouzelis N. The orthodontic-periodontic interrelationship in integrated treatment challenges: A systematic review. J Oral Rehabil 2010;37(5):377–390. DOI: 10.1111/j.1365-2842.2010.02068.x.
3. Oktaviani V, Santoso O. Perbedaan Indeks Higiene Oral dan pH Plak Kelompok Pemakai dan Bukan Pemakai Pesawat Ortodonti Cekat 2016;5(1):8.
4. Bowen WH, Burne RA, Wu H, et al. Oral biofilms: pathogens, matrix and polymicrobial interactions in microenvironments. Trends Microbiol 2018;26(3):229–242. DOI: 10.1016/j.tim.2017.09.008.
5. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence 2011;2(5):435–444. DOI: 10.4161/viru.2.5.16140.
6. Verrusio C, Iorio-Siciliano V, Blasi A, et al. The effect of orthodontic treatment on periodontal tissue inflammation: a systematic review. Quintessence Int 2018;49(1):69–77. DOI: 10.3290/j.qi.a39225.
7. Perkowski K, Baltaza W, Conn DB, et al. Examination of oral biofilm microbiota in patients using fixed orthodontic appliances in order to prevent risk factors for health complications. Ann Agric Environ Med 2019;26(2):231–235. DOI: 10.26444/aaem/105797.
8. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. Curr Opin Microbiol 2015;23:141–147. DOI: 10.1016/j.mib.2014.11.013.
9. Rickard AH, Gilbert P, High NJ, et al. Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol 2003;11(2):94–100. DOI: 10.1016/s0966-842x(02)00034-3.
10. Thurnheer T, Karygianni L, Flury M, et al. Fusobacterium species and subspecies differentially affect the composition and architecture of supra- and subgingival biofilms models. Front Microbiol 2019;10:1716. DOI: 10.3389/fmicb.2019.01716.
11. Chew J, Zilm PS, Fuss JM, et al. A proteomic investigation of Fusobacterium nucleatum alkaline-induced biofilms. BMC Microbiol 2012;12(1):189. DOI: 10.1186/1471-2180-12-189.
12. Kaplan CW, Lux R, Haake SK, et al. The Fusobacterium nucleatum outer membrane protein RadD is an arginine-inhibitable adhesin required for inter-species adherence and the structured architecture of multi-species biofilm. Mol Microbiol 2009;71(1):35–47. DOI: 10.1111/j.1365-2958.2008.06503.x.
13. Cochrane K, Robinson AV, Holt RA, et al. A survey of Fusobacterium nucleatum genes modulated by host cell infection. Microb Genom 2020;6(2):e000300. DOI: 10.1099/mgen.0.000300.
14. Kaplan A, Kaplan CW, He X, et al. Characterization of aid1, a novel gene involved in Fusobacterium nucleatum interspecies interactions. Microb Ecol 2014;68(2):379–387. DOI: 10.1007/s00248-014-0400-y.
15. Liu P, Liu Y, Wang J, et al. Detection of Fusobacterium nucleatum and fadA adhesin gene in patients with orthodontic gingivitis and non-orthodontic periodontal inflammation. PLOS One 2014;9(1):e85280. DOI: 10.1371/journal.pone.0085280.
16. Brennan CA, Garrett WS. Fusobacterium nucleatum – symbiont, opportunist and oncobacterium. Nat Rev Microbiol 2019;17(3):156–166. DOI: 10.1038/s41579-018-0129-6.
17. Wu T, Cen L, Kaplan C, et al. Cellular components mediating coadherence of Candida albicans and Fusobacterium nucleatum. J Dent Res 2015;94(10):1432–1438. DOI: 10.1177/0022034515593706.
18. Wang Q-Q, Zhang C-F, Chu C-H, et al. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. Int J Oral Sci 2012;4(1):19–23. DOI: 10.1038/ijos.2012.17.
19. Najafi K, Gholizadeh P, Tanomand A, et al. Oral cavity infection by Enterococcus faecalis: virulence factors and pathogenesis. Rev Med Microbiol 2019;30(2). DOI: 10.1097/MRM.0000000000000168.
20. Ch'ng J-H, Chong KKL, Lam LN, et al. Biofilm-associated infection by enterococci. Nat Rev Microbiol 2019;17(2):82–94. DOI: 10.1038/s41579-018-0107-z.
21. Anderson AC, Jonas D, Huber I, et al. Enterococcus faecalis from food, clinical specimens, and oral sites: prevalence of virulence factors in association with biofilm formation. Front Microbiol 2016;6:1534. DOI: 10.3389/fmicb.2015.01534.
22. Stinemetz EK, Gao P, Pinkston KL, et al. Processing of the major autolysin of E. faecalis, AtlA, by the zinc-metalloprotease, GelE,

impacts AtlA septal localization and cell separation. PLoS One 2017;12(10):e0186706.
23. Guiton PS, Hung CS, Kline KA, et al. Contribution of autolysin and sortase A during Enterococcus faecalis DNA-dependent biofilm development. Infect Immun 2009;77(9):3626–3638. DOI: 10.1128/IAI.00219-09.
24. Paganelli FL, Willems RJL, Jansen P, et al. Enterococcus faecium biofilm formation: identification of major autolysin AtlAEfm, associated Acm surface localization, and AtlAEfm-independent extracellular DNA release. mBio 2013;4(2):e00154. DOI: 10.1128/mBio.00154-13.
25. Putri MH, Herijulianti E. Ilmu Pencegahan Penyakit Jaringan Keras dan Jaringan Pendukung Gigi | OPAC Integrasi | Online Public Access Catalog | Universitas Gadjah Mada. RGC; 2010.
26. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: A persistent public health problem. Clin Microbiol Rev 2007;20(1):133–163. DOI: 10.1128/CMR.00029-06.
27. Garcia MC, Lee JT, Ramsook CB, et al. A role for amyloid in cell aggregation and biofilm formation. PLoS One 2011;6(3):e17632. DOI: 10.1371/journal.pone.0017632.
28. Nobile CJ, Mitchell AP. Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Curr Biol 2005;15(12):1150–1155. DOI: 10.1016/j.cub.2005.05.047.
29. Mulhern SM, Logue ME, Butler G. Candida albicans transcription factor Ace2 regulates metabolism and is required for filamentation in hypoxic conditions. Eukaryot Cell 2006;5(12):2001–2013. DOI: 10.1128/EC.00155-06.
30. Saputo S, Kumar A, Krysan DJ. Efg1 directly regulates ACE2 expression to mediate cross talk between the cAMP/PKA and RAM pathways during Candida albicans morphogenesis. Eukaryot Cell 2014;13(9):1169–1180. DOI: 10.1128/EC.00148-14.
31. Widyarman AS, Bachtiar EW, Bachtiar BM, et al. Inhibitory effect of probiotic lactobacilli against Streptococcus mutans and Porphyromonas gingivalis biofilms. Sci Dental J 2019;3(2):50. DOI: 10.4103/SDJ.SDJ_8_19.
32. Widyarman AS, Ramajayanti T, Theodorea CF. Indonesian strain of probiotic reduces the initial biofilm colonization. The Open Dent J 2020;14(1). DOI: 10.2174/1874210602014010544.
33. Widyarman AS, Theodorea CF. Novel indigenous probiotic Lactobacillus reuteri strain produces anti-biofilm reuterin against pathogenic periodontal bacteria. Eur J Dent 2021;16(1):96–101. DOI: 10.1055/s-0041-1731591.
34. Mu Q, Tavella VJ, Luo XM. Role of Lactobacillus reuteri in human health and diseases. Front Microbiol 2018;9:757. DOI: 10.3389/fmicb.2018.00757.
35. Sari Widyarman A, Kiky Elysia Lazaroni N. Persistent endodontics pathogens biofilm inhibited by Lactobacillus reuteri Indonesian strain. J Dent Indones 2019;26(3). Available from: https://scholarhub.ui.ac.id/jdi/vol26/iss3/7/.
36. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol 2008;226(5):1065–1073.
37. Julious SA. Sample size of 12 per group rule of thumb for a pilot study. Pharm Stat 2005;4:287–291. DOI: 10.1002/pst.185.
38. Johanson GA, Brooks GP. Initial scale development: Sample size for pilot studies. Educ Psychol Meas 2010;70:394–400. DOI: 10.1177/0013164409355692.
39. Nikoomanesh F, Roudbarmohammadi S, Roudbary M, et al. Investigation of bcr1 gene expression in Candida albicans isolates by RT-PCR technique and its impact on biofilm formation. iem 2016;2(1):22–24. DOI: 10.7508/iem.2016.01.007.
40. Ranjith K, Kalyana Chakravarthy S, Adicherla H, et al. Temporal expression of genes in biofilm-forming ocular *Candida albicans* isolated from patients with keratitis and orbital cellulitis. Invest Ophthalmol Vis Sci 2018;59(1):528–538. DOI: 10.1167/iovs.17-22933.
41. Nailis H, Coenye T, Van Nieuwerburgh F, et al. Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. BMC Mol Biol 2006;7:25. DOI: 10.1186/1471-2199-7-25.
42. Vos M, Quince C, Pijl AS, et al. A Comparison of rpoB and 16S rRNA as markers in pyrosequencing studies of bacterial diversity. PLoS One 2012;7(2):e30600. DOI: 10.1371/journal.pone.0030600.
43. Wang L, Dong M, Zheng J, et al. Relationship of biofilm formation and gelE gene expression in Enterococcus faecalis recovered from root canals in patients requiring endodontic retreatment. J Endod 2011;37(5):631–636. DOI: 10.1016/j.joen.2011.02.006.
44. Zheng J-X, Wu Y, Lin Z-W, et al. Characteristics of and virulence factors associated with biofilm formation in clinical Enterococcus faecalis isolates in China. Front Microbiol 2017;8:2338. DOI: 10.3389/fmicb.2017.02338.
45. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. J Oral Microbiol 2011;3. DOI: 10.3402/jom.v3i0.5771.
46. Khanpayeh E, Jafari AA, Tabatabaei Z. Comparison of salivary Candida profile in patients with fixed and removable orthodontic appliances therapy. Iran J Microbiol 2014;6(4):263–268. PMID: 25802710.
47. Widyarman AS, Hartono V, Marjani LI, et al. Lactobacillus reuteri containing probiotic Lozenges consumption reduces Streptococcus mutans, Streptococcus sobrinus, Porphyromonas gingivalis, and Aggregatibacter actinomycetemcomitans in orthodontic patients. J Int Dent Med Res 2018;11(2):628–633.
48. Restrepo M, Bussaneli DG, Jeremias F, et al. Control of white spot lesions with use of fluoride varnish or chlorhexidine gel during orthodontic treatment A randomized clinical trial. J Clin Pediatr Dent 2016;40(4):274–280. DOI: 10.17796/1053-4628-40.4.274.
49. Lu H, Tang H, Zhou T, et al. Assessment of the periodontal health status in patients undergoing orthodontic treatment with fixed appliances and Invisalign system. Medicine (Baltimore) 2018;97(13):e0248. DOI: 10.1097/MD.0000000000010248.
50. Fitriasari N, Wahyuni IS. POTENSI PROBIOTIK DALAM TATALAKSANA ORAL CANDIDIASIS: Ulasan Sistematik. Odonto : Dent J 2021;8(1):34–44. DOI: 10.30659/odj.8.1.34-44.
51. Guo L, Feng Y, Guo H-G, et al. Consequences of orthodontic treatment in malocclusion patients: clinical and microbial effects in adults and children. BMC Oral Health 2016;16(1):112. DOI: 10.1186/s12903-016-0308-7.
52. Alqerban A. Efficacy of antimicrobial photodynamic and photobiomodulation therapy against Treponema denticola, fusobacterium nucleatum and human beta defensin-2 levels in patients with gingivitis undergoing fixed orthodontic treatment: a clinic-laboratory study. Photodiagnosis Photodyn Ther 2020;29:101659.
53. Willett JLE, Ji MM, Dunny GM. Exploiting biofilm phenotypes for functional characterization of hypothetical genes in Enterococcus faecalis. NPJ Biofilms Microbiomes 2019;5(1):23. DOI: 10.1038/s41522-019-0099-0
54. Sinaga E. Regulasi Ekspresi Gen. Regulasi Ekspresi Gen 2011;1–24.
55. Van Peer G, Mestdagh P, Vandesompele J. Accurate RT-qPCR gene expression analysis on cell culture lysates. Sci Rep 2012;2:222. DOI: 10.1038/srep00222.
56. Shemesh M, Tam A, Steinberg D. Expression of biofilm-associated genes of Streptococcus mutans in response to glucose and sucrose. J Med Microbiol 2007;56(11):1528–1535. DOI: 10.1099/jmm.0.47146-0.
57. Fanning S, Xu W, Solis N, et al. Divergent targets of Candida albicans biofilm regulator Bcr1 in vitro and in vivo. Eukaryot Cell 2012;11(7):896–904. DOI: 10.1128/EC.00103-12.
58. Matsubara VH, Bandara HMHN, Mayer MPA, et al. Probiotics as antifungals in mucosal candidiasis. Clin Infect Dis 2016;62(9):1143–1153. DOI: 10.1093/cid/ciw038.
59. Li Q, Tan L, Wang H, et al. Fusobacterium nucleatum interaction with Pseudomonas aeruginosa induces biofilm-associated antibiotic tolerance via Fusobacterium adhesin A. ACS Infect Dis 2020;6(7):1686–1696. DOI: 10.1021/acsinfecdis.9b00402.
60. Kaplan A. Identification and characterization of a novel lipoprotein in Fusobacterium nucleatum. UCLA; 2015 Available from: https://escholarship.org/uc/item/3r90n33c.
61. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402–408.

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62. Barzegari A, Kheyrolahzadeh K, Hosseiniyan Khatibi SM, et al. The battle of probiotics and their derivatives against biofilms. Infect Drug Resist 2020;13:659–672. DOI: 10.2147/IDR.S232982.
63. Guneser MB, Eldeniz AU. The effect of gelatinase production of Enterococcus faecalis on adhesion to dentin after irrigation with various endodontic irrigants. Acta Biomater Odontol Scand 2016;2(1):144–149. DOI: 10.1080/23337931.2016.1256212.
64. Hashem YA, Amin HM, Essam TM, et al. Biofilm formation in enterococci: Genotype-phenotype correlations and inhibition by vancomycin. Sci Rep 2017;7(1):5733. DOI: 10.1038/s41598-017-05901-0.
65. Ali L, Goraya MU, Arafat Y, et al. Molecular mechanism of quorum-sensing in Enterococcus faecalis: its role in virulence and therapeutic approaches. Int J Mol Sci 2017;18(5):960. DOI: 10.3390/ijms18050960.
66. Bonifait L, Chandad F, Grenier D. Probiotics for oral health: myth or reality? J Can Dent Assoc 2009;75(8):585–590. PMID: 19840501
67. Urbańska M, Szajewska H. The efficacy of Lactobacillus reuteri DSM 17938 in infants and children: A review of the current evidence. Eur J Pediatr 2014;173(10):1327–1337. DOI: 10.1007/s00431-014-2328-0.
68. Benic GZ, Farella M, Morgan XC, et al. Oral probiotics reduce halitosis in patients wearing orthodontic braces: A randomized, triple-blind, placebo-controlled trial. J Breath Res 2019;13(3):036010. DOI: 10.1088/1752-7163/ab1c81.
69. Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, et al. Probiotic mechanisms of action. Ann Nutr Metab 2012;61(2):160–174. DOI: 10.1159/000342079.
70. Widyarman AS, Yunita ST, Prasetyadi T. Consumption of yogurt containing probiotic *Bifidobacterium lactis* reduces *Streptococcus mutans* in orthodontic patients. Sci Dent J2018;2(1):19–25. DOI: 10.26912/sdj.v2i1.1913.
71. Alp S, Baka ZM. Effects of probiotics on salivary *Streptecoccus mutans* and Lactobacillus levels in orthodontic patients. Am J Orthod Dentofacial Orthop 2018;154(4):517–523. DOI: 10.1016/j.ajodo.2018.01.010.
72. Goyal N, Shamanna PU, Varughese ST, et al. Effects of amine fluoride and probiotic mouthwash on levels of *Porphyromonas gingivalis* in orthodontic patients: a randomized controlled trial. J Indian Soc Periodontol 2019;23(4):339–344. DOI: 10.4103/jisp.jisp_551_18.

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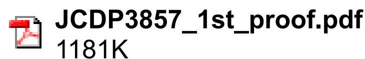

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ORIGINAL RESEARCH

Lactobacillus Reuteri Probiotic Consumption Reduced Various Virulence Gene Expression in Dental Plaque of Fixed Orthodontic Subjects

Joko Kusnoto[1], Siti Sara Safirah[2], Litayana Ria Anggriani Sitorus[3], Winnie Valentini[4], Armelia Sari Widyarman[5]

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Abstract

Aims: The aim of this study was to determine the effect of consuming lozenges containing *Lactobacillus reuteri* probiotic Prodentis lozenges on the expression of *BCR1, ACE2*, *fadA, aid1*, *gelE*, and *atlA* genes in dental biofilms of subjects using fixed orthodontic appliances.

Materials and methods: Plaque samples ($n = 20$) obtained from a previous study were used in this research. Each subject consumed *L. reuteri* probiotic lozenges (2×10^8 CFU/mL) each day for 2 weeks. RNA was extracted from the samples and synthesized into cDNA. The expression of the gene transcription factors *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes in biofilms of subjects who used fixed orthodontic appliances was detected using real-time quantitative polymerase chain reaction (RT-qPCR).

Results: The expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes were decreased after consuming the *L. reuteri* probiotic lozenges for 2 weeks ($p < 0.05$).

Conclusion: Consuming *L. reuteri* probiotic lozenges would affect the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* in plaque from patients using fixed orthodontic appliances. By reducing the expression of the virulence genes, bacterial number would be reduced and biofilm production can also be reduced.

Clinical significance: Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression. Routine consumption of probiotic lozenges can help reduce potential bacterial infection and increase the oral health of patients using fixed orthodontic appliances.

Keywords: Biofilm, Gene expression, *Lactobacillus reuteri*, Orthodontic, Probiotic.

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Introduction

Orthodontic treatment is common today in the community. Among adults and children, orthodontic treatment may be undertaken for dental care or esthetic reasons.[1] Orthodontic treatment using fixed appliances aims to ensure proper occlusion and esthetic function, with appropriate tooth movement. Fixed orthodontic treatment can lead to changes in the oral environment and oral flora composition and an increase in the amount of plaque due to difficulty in maintaining oral hygiene.[2–5] In addition, excess composite around the base of the bracket that used in orthodontic treatment is an important factor that can cause plaque accumulation due to the presence of rough surfaces and cracks on the enamel composite surfaces.[3–5] Biofilm accumulation on teeth and soft tissues in the oral cavity can lead to caries, gingivitis, and periodontitis.[6,7]

Fusobacterium nucleatum is a dominant bacterial species that plays an important role in the formation of dental biofilms and periodontal tissue disease. In the formation of biofilms, *F. nucleatum* being a “bridging” or “linking” organism between initial bacterial colonization and final bacterial colonization, which are unable to bind to each other directly. *F. nucleatum* can also co-aggregate with various microbial species in the oral cavity.[8–10] *F. nucleatum* encodes several adhesion genes involved in interspecies interactions, including fusobacterium adhesion A (*fadA*), fusobacterial outer membrane protein A (*fomA*), *radD* (an arginine-inhibitable adhesin), and adherence inducing determinant gene 1 (*aid1*).[8,11–14] Fusobacterium adhesin A (FadA) is known to be involved in *F. nucleatum* invasion and adhesion to host cells and is highly conserved among oral Fusobacterium species.[8,15,16] FadA has been identified has a major virulence factor in *F. nucleatum* in interspecies interactions with *Streptococcus* mediated by *radD,* as it increases the binding specificity of *F. nucleatum* to other microbial species.[14] The arginine-inhibitable adhesion *radD* is required by *F. nucleatum* for co-adherence with various species of Gram-positive bacteria, such as streptococci (early colonizers), and fungal species, such as *Candida*.[11,12,17]

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Enterococcus faecalis (*E. faecalis*) is associated with chronic periodontitis and chronic apical periodontitis in failed root canal treatment.[18] *E. faecalis* is a Gram-positive aerobic bacterium. The severity of *E. faecalis* infection depends on the immune response and virulence factors, which can exacerbate infection and play a role in increasing biofilm formation.[19] There are several genes associated with *E. faecalis* biofilm formation, including gelatinase (*gelE*) and autolysin (*atlA*).[20] GelE in *E. faecalis* plaque or saliva isolates showed resistance to antibiotics and high biofilm formation ability.[21] atlA is the main peptidoglycan hydrolase or autolysin of *E. faecalis*.[22] AtlA plays a role in the biofilm maturation stage during which extracellular DNA (eDNA) is released and contributes to biofilm attachment and stability.[23,24]

An increase in the number of colonies of microorganisms also increased, one of which was *Candida albicans*, which causes infections of oral mucosa.[25,26] *C. albicans* has a protein in the form of an adhesive that mediates other microorganisms to adhere to abiotic and host surfaces to form biofilms.[27] Several *C. albicans* gene transcription factors, including biofilm and cell wall regulator 1 (*BCR1*) and angiotensin converting enzyme 2 (*ACE2*), play a role in the formation of biofilms. BCR1 acts as a major regulator of *C. albicans* biofilm formation.[28] The ACE2 transcription factor plays a role in fungal adherence, biofilm formation, and hyphal morphogenesis. In addition, ACE2 plays a role in regulating the expression of genes involved in cell wall separation and metabolism.[29] As shown in previous research, ACE2 is required for filamentation, and it can increase the number of pseudohyphae cells at the time of biofilm formation.[30]

Biofilm formation plays a role in increasing antibiotic resistance in bacterial cells. Therefore, an effective therapy is needed to prevent biofilm formation. The use of probiotics has been suggested as a promising approach to prevent and treat microbial diseases and biofilm activity in the oral cavity.[31,32] Several studies have proven that the use of probiotics has oral cavity health benefits, such as preventing caries and periodontal disease.[32,33] One commercial probiotic proven to be beneficial for oral health is *Lactobacillus reuteri*. The antimicrobial activity of *L. reuteri* inhibits colonization by pathogenic microbes and interacts the inhibition directly with host cells.[34] *L. reuteri* also inhibits the growth of *C. albicans* and *E. faecalis* biofilms.[35,36] However, no studies have investigated the effect of the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in dental plaque biofilms found in patient's oral environment with fixed orthodontic appliances. By analyzing the virulence genes found in several pathogenic bacteria, we can hypothesize that the downregulation of these genes will lead to less biofilm production and healthier oral health conditions during the duration of fixed orthodontic appliances usage. Thus, to bridge the knowledge gap, the aim of this study was to determine the effect of consuming lozenges containing the probiotic *L. reuteri* on the expression of *BCR1*, *ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes in biofilms from subjects using fixed orthodontic appliances.

Materials and Methods

Sample Collection

Subjects undergoing fixed orthodontic therapy were enrolled in this open-label prospective, clinical trial. Ethical approval for the study was granted by the Institutional Review Board of the Faculty of Dentistry, Universitas Indonesia (approval number: 100,701,020). The inclusion criteria specified participants aged 18 years or older who had been undergoing orthodontic treatment with fixed appliances for a minimum duration of 1 year and had not consumed probiotics or antibiotics in the preceding 3 months. The fixed orthodontic appliances used by the subjects are conventional metal braces fixed appliance. Exclusion criteria encompassed individuals with systemic conditions such as hypertension or diabetes, those on systemic medications including antihypertensives, analgesics, hormonal therapies, sedatives, or anti-seizure medications, as well as individuals presenting with severe periodontal disease or known allergies to probiotics.

The study utilized *L. reuteri* Prodentis lozenges, obtained from BioGaia (Stockholm, Sweden). Each lozenge (800 mg) contained a minimum of 2×10^8 live *Limosilactobacillus reuteri* Prodentis (previously classified as *L. reuteri*). This food supplement, specifically formulated for oral health, included a proprietary combination of the *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289 strains, which are recognized for their potential to support and maintain oral health.

To standardize oral hygiene practices, all participants were provided with a standardized toothbrush and toothpaste for use throughout the study period. Participants received oral hygiene instructions and were instructed to brush their teeth twice daily. Each participant was administered one probiotic lozenge containing *Limosilactobacillus reuteri* daily for 14 days, to be taken once per day after morning toothbrushing and prior to breakfast. Plaque samples were collected from participants on the day of enrolment, prior to the initiation of probiotic lozenge consumption, and again on the 14th day following the completion of the probiotic regimen.

Before sample collection, mandatory rapid antigen test for SARS-CoV-2 detection had to be taken by the subjects in response to the COVID-19 pandemic (as of 30 September 2020 when collecting the samples) and the test result had to be negative for the subject partaking in the study. In brief, the participants were instructed not to eat or drink anything 2 hours prior to collection of sample. Samples were collected using sterile cotton buds and swabbed from buccal/mesial/distal/lingual/occlusal surfaces of the index teeth of the subjects. The plaque samples were stored in sterile falcon tubes with 5 mL of phosphate-buffered saline (PBS).

Furthermore, clinical data, including the Oral Hygiene Index-Simplified score and the Papilla Bleeding Index, were recorded at each visit. Overall, 20 subjects had enrolled and fulfilled all requirements and inclusion criteria, as 20 subjects were proved to be sufficient to provide valid data as a pilot study.[37,38] Plaque samples from the subjects were continued unto the following downstream analysis.

RNA Extraction, cDNA Synthesis, and Quantification

RNA from the sample was extracted using TRIzol reagent methodology as instructed by the manufacturer (Thermo Fisher, Waltham, MA). The extracted RNA was synthesized into cDNA using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The mixture for cDNA synthesis is described in Table 1. The cDNA was then quantified using an Invitrogen™ Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). The cDNA was stored at –20°C for storage of directly used for downstream analysis.

qPCR Analysis

Amplification and detection by qPCR (Applied Biosystems, Waltham, MA) were performed. The components of the qPCR Master Mix are listed in Table 2. Using a specific kit, namely HOT FIREpol EvaGreen® qPCR Mix (Solis Biodyne, Tartu, Estonia) which was activated by

Table 1: Reagent components for DNase I reaction solution

Component	*Volume*
4 × DN Master Mix	2 mL
RNA template	0.5 pg – 0.5 mg
Nuclease-free water	X mL
Total volume	8 mL

Table 2: Components of Master Mix RT-qPCR

Component	*Volume*
5 × HOT FIREPol EvaGreen® qPCR Mix Plus	4 mL
Primer *Forward*	1 mL
Primer *Reverse*	1 mL
DNA *template*	2 mL
NFW	12 mL
Total	20 mL

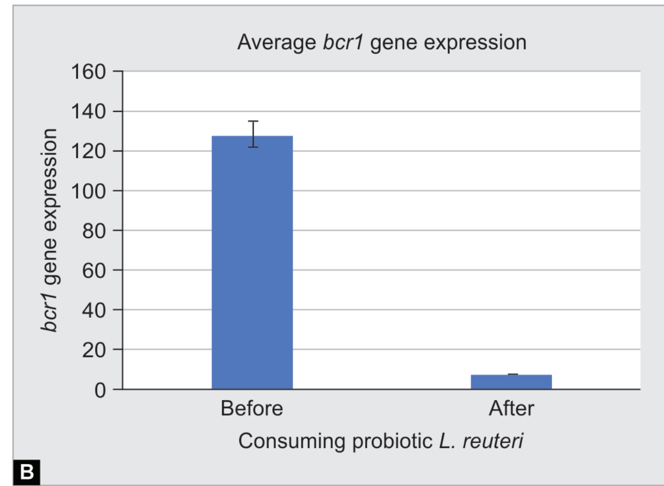
incubation at 95°C for 10 minutes. This was followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing temperature at 60–65°C (Table 3), and elongation at 72°C for 20 seconds. For templates longer than 150 bp, the annealing and elongation times were extended to 30 seconds. Actin gene encoding as the housekeeping gene was used for normalization purposes. qPCR was performed on cDNA. qPCR was performed using the primers listed in Table 3.

Data Analysis and Outcome Analysis

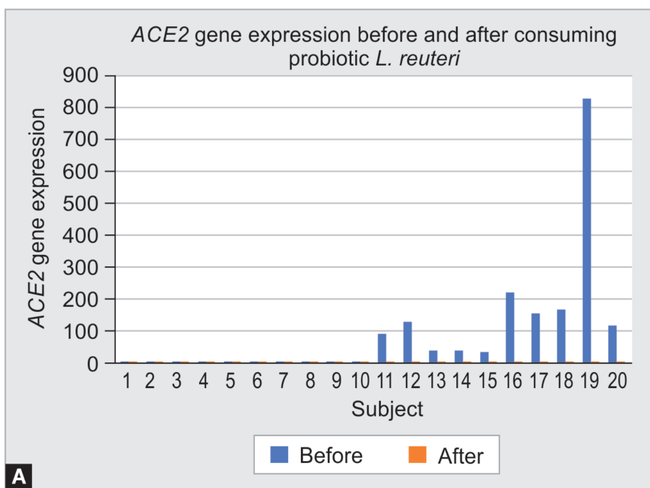
The data were analyzed using the Shapiro–Wilk normality test ($p > 0.05$). For data with a normal distribution, a paired *t*-test was applied ($p < 0.05$). The software used for the analysis is Statistical Package for the Social Sciences (SPSS) version 27 (IBM, Armonk, NY). The outcome analysis are the quantitative data presented from the RT-qPCR instrument. This data can then be correlated with previous study that also conducted such research to ensure the validity of this data.

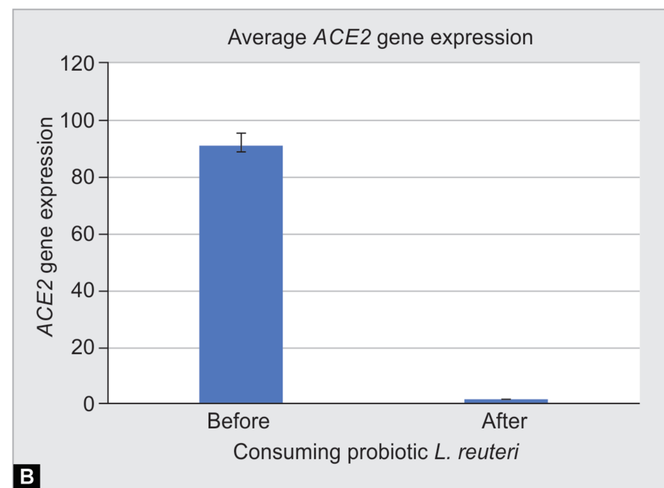
Table 3: *Primer sequence* for RT-qPCR

Genes	*Primer sequence*	*Temperature and cycle settings*
BCR1[39]	*forward*: 5′-CTTCAGCAGCTTCATTAACACCTA-3′ *reverse*: 5′-TCTTGGATCAGGTGTACTTTTCAA-3′	Initial denaturation of 95°C for 5 minutes; 40 cycles of denaturation at 95°C for 1 minute and annealing at 58°C for 1 minute.
ACE2[40]	*forward* 5′-AGAATTGACCGTTGTCCGTGTAAG-3′ *reverse*: 5′-AATGGGTGAATAAATCCCTCCCTAA-3′	Initial denaturation 95°C for 2 minutes; 40 cycles of denaturation at 95°C for 30 seconds and annealing at 60°C for 1 minute.
Housekeeping gene C. albicans: *ACT1*[41]	*forward*: 5′-TTTCATCTTCTGTATCAGAGGAACTTATTT-3′ *reverse*: 5′-ATGGGATGAATCATCAAACAAGAG-3′	Initial denaturation 95°C for 10 minutes; 40 cycles of denaturing 95°C for 15 seconds and annealing 60°C for 1 minute.
fadA[15]	*forward*: 5′-CAC AAG CTG ACG CTG CTA GA- 3′ *reverse*: 5′-TTA CCA GCT CTT AAA GCT TG-3′	Initial incubation for 4 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55.8°C for 30 seconds, and elongation at 72°C for 40 seconds and the final elongation for 6 minutes.[14]
aid1[14]	*forward:* 5′-TACAGGAG GTGCCGTAGCAG-3′ *reverse:* 5′-TTTTTGTTAATTCT CCAGCTCCA-3′	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.[13]
Housekeeping gene *F. nucleatum*: *rpoB*[42]	*forward:* 5′-GGYTWYGAAGTNCGHGACGTDCA-3′ *reverse:* 5′-TGACGYTGCATGTTBGMR CCCATMA-3′	Initial incubation for 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and elongation at 60°C for 1 minute.
gelE[43]	*forward:* 5′-CGGAACATACTGCCGGTTTAGA-3′ *reverse:* 5′-TGGATTAGATGCACCCGAAAT -3′	Initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 5 seconds, and annealing at 60°C for 30 seconds.
atlA[44]	*forward:* 5′-AATAATCAATCAGGAACGAATACG-3′ *reverse:* 5′-GCCACACTAACACCGAAT-3′	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.
Housekeeping gene E. faecalis: rpoA[44]	*forward:* 5′-GTGAAACCTGGTCGTGGCTA-3′ *reverse:* 5′-CGACGAACGGGTGTGTAGAT-3′	Initial denaturation at 95°C for 2 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 60 seconds.



Figs 1A and B: (A) A graph showing *bcr1* gene expression in plaque samples ($N = 20$) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *bcr1* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges





Figs 2A and B: (A) A graph showing *ACE2* gene expression in plaque samples ($N = 20$) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *ACE2* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

Results

Based on the results of the qPCR test, the expression of *BCR1* (Fig. 1), *ACE2* (Fig. 2), *fadA* (Fig. 3), *aid1* (Fig. 4), *gelE* (Fig. 5), and *atlA* (Fig. 6), on average, were decreased when comparing the day-0 prior to probiotic consumption and day-14 after the subjects consumed the probiotic *L. reuteri*.

As shown by the results of the Shapiro–Wilk normality test, all the data were normally distributed ($p > 0.05$). The data were analyzed using a paired *t*-test, with a significance level of $p < 0.05$. The results of the paired *t*-test revealed a significant difference in the comparison of the *BCR1* and *ACE2* gene expression data. There was a significant difference ($p < 0.05$) in the expression of *BCR1, ACE2*, *fadA*, *aid1*, *gelE*, and *atlA* genes after consuming the probiotic *L. reuteri*.

Discussion

The use of fixed orthodontic appliances often causes poor oral hygiene, thus facilitating microorganism accumulation and various pathological conditions in the oral cavity, such as fungal infections.[45] A previous study revealed an increase in *Candida* in saliva after using fixed orthodontic appliances. The authors attributed this to the design of fixed orthodontic appliances, which creates a space for the retention of food waste.[46] Thus, patients must be instructed about good oral hygiene practices after orthodontic treatment.[38] Fixed orthodontic appliances also induce changes in buffer capacity, salivary flow rates, and acidity (pH), leading to plaque accumulation and an increase in caries and periodontal disease.[47–49] Based on this information, we attempted this study to prove the benefits of consuming probiotics lozenges as a supplement for patients using fixed orthodontic appliances.

Biofilm formation is an important virulence factor of *F. nucleatum* due to its higher resistance to host defense or antibacterial agents compared to planktonic cells.[50] Several studies detected increased numbers of *Porphyromonas gingivalis, F. nucleatum, P. intermedia,* and *Tannerella forsythia* after the use of fixed orthodontic appliances.[15,51,52] They also reported that *F. nucleatum* increased

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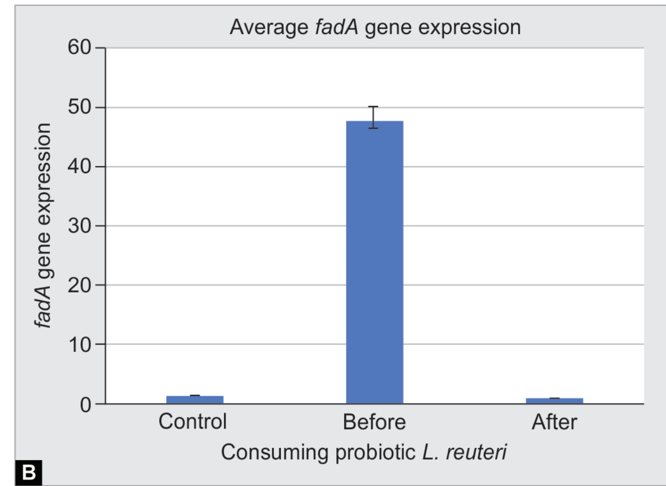
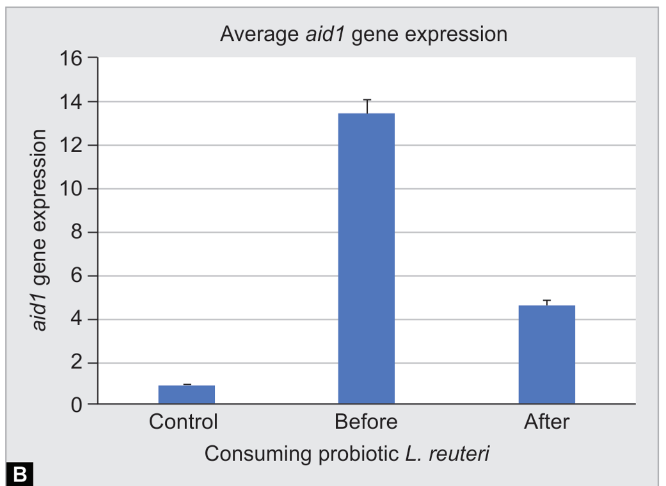
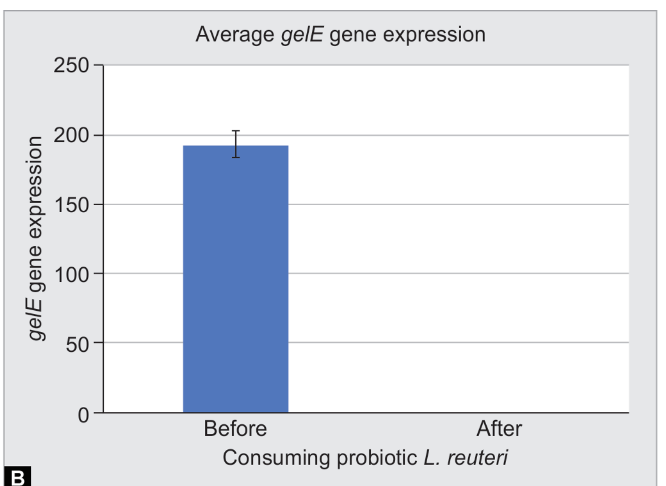


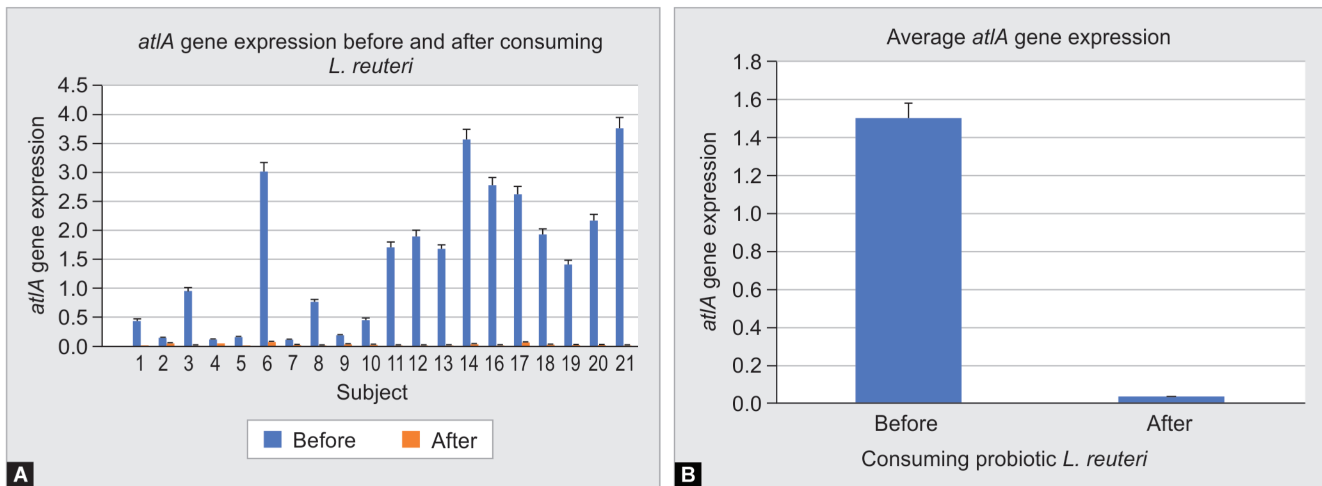
Figs 3A and B: (A) A graph showing *fadA* gene expression in plaque samples ($N = 20$) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *fadA* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges



Figs 4A and B: (A) A graph showing *aid1* gene expression in plaque samples ($N = 20$) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *aid1* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges



Figs 5A and B: (A) A graph showing *gelE* gene expression in plaque samples ($N = 20$) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *gelE* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges



Figs 6A and B: (A) A graph showing *atlA* gene expression in plaque samples ($N = 20$) as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges; (B) A graph showing the average of *atlA* gene expression in plaque samples as assessed by the RT-qPCR method and the formation of *C. albicans* biofilms before and after consuming *L. reuteri* probiotic lozenges

the risk of periodontitis in orthodontic patients due to a conducive environment for anaerobic bacteria.[15,51,52] Biofilm formation is also a contributing factor to *E. faecalis* colonization and infection. Biofilms develop through various processes by which bacteria adhere to surfaces, decompose complex matrices, and develop into bacterial colonies, which adhere to surfaces.[53]

In this study, we used the qPCR method and $2^{-\Delta\Delta CT}$ formula to calculate target gene expression. As shown by the results, the probiotic *L. reuteri* affected the expression of *BCR1, ACE2, fadA, aid1, gelE*, and *atlA* genes and the formation of *C. albicans, F. nucleatum*, and *E. faecalis* biofilms. Based on the Shapiro–Wilk normality test, all the data were normally distributed, with $p > 0.05$. The paired *t*-test results revealed significant differences in the expression of *BCR1, ACE2, fadA, aid1, gelE*, and *atlA* genes (all $p < 0.05$).

Based on the results of this study, the probiotic *L. reuteri* significantly downregulated the transcription of *BCR1, ACE2, fadA, aid1, gelE*, and *atlA* genes. In gene expression, the process of translating genetic information in the form of sequence of bases of DNA or RNA into proteins.[54] Through gene expression measurement, it is possible to assess qualitatively and quantitatively the effect of a treatment, such as the administration of a drug compound.[55] Gene expression in microorganisms is involved in regulating cell-cell communication, carbohydrate metabolism, adherence, and adaptation to the surrounding environment. A decrease in the expression of specific genes can reduce microorganism colonization and microorganism numbers.[56]

BCR1, a major gene transcription factor, produces an adhesin protein, which facilitates *C. albicans* attachment to mucosal surfaces, which is a critical stage of infection.[39] Deletion of *BCR1* eliminates *C. albicans* gene function, resulting in a decrease in biofilm formation.[57] This was supported by the results of the present study, which revealed a statistically significant decrease in *BCR1* gene expression after consuming the probiotic.

The expression of *ACE2* also decreased based on the results of the statistical tests. With deletion of *ACE2*, *C. albicans* is unable to form hyphal cells, and thus biofilm formation is inhibited.[30] An earlier in vitro study showed that probiotics have antifungal effects against *C. albicans* in the oral cavity. Regular use of probiotics helped to inhibit *Candida* biofilms and reduced *Candida* colonization in the oral cavity, thereby reducing the possibility of candidiasis infection.[58]

FadA protein is the main *F. nucleatum* virulence factor and mediates microbial attachment and colonization.[8] Based on the results of this study, the probiotic *L. reuteri* appears to influence the pathogenicity of *F. nucleatum* adhesion molecules and colonization and affect biofilm formation through decreased expression of the *fadA* gene.[35] Various *F. nucleatum* adhesins mediate adhesion and aggregation and function as coaggregation intermediaries in the formation and maturation of dental biofilms.[59] The interaction of Fusobacterium with other species is largely mediated by the adhesin genes *radD* and *aid1*.[60] The *aid1* gene plays a role in interspecies interactions, colonization, and aggregation of *F. nucleatum*. In a previous study, inactivation of the *aid1* gene decreased the ability of *F. nucleatum* to aggregate, especially with *Streptococcus* spp. or *E. faecalis*.[14] As shown in earlier studies, probiotics can affect the expression of genes involved in cell adhesion, quorum sensing (QS), virulence factors, and biofilm formation.[61,62]

The *E. faecalis gelE* gene has the ability to hydrolyse gelatine, collagen, fibrin, and other peptides.[63] Gene *gelE* is a virulence factor in infection formation through bacterial attachment and biofilm formation.[22] In *E. faecalis*, biofilm formation is regulated by QS, where Fsr regulates the expression of the *gelE* gene.[64] Fsr regulates the formation of *E. faecalis* biofilms through its product *gelE* and serine proteases.[43] QS is a molecular mechanism by which bacterial cells communicate with each other via signaling molecules in biofilms. If the protease encoded by a signaling factor is decreased, communication between bacteria and biofilm formation will be disrupted.[62] GelE can also activate *atlA*, which is responsible for eDNA release at the biofilm maturation stage.[65]

AtlA is involved in the hydrolysis of peptidoglycan, which plays an important role in separating cells division after replication.[22] AtlA plays a role in the biofilm maturation stage of *E. faecalis*, during which eDNA is released and contributes to biofilm attachment and stability, biofilm defects in primary attachment, and decreased biofilm production.[20,23] This study focused on the probiotic *L. reuteri*, which has ability to secrete antimicrobial substances and compete with oral pathogens for adhesion to mucosa. In addition, *L. reuteri* can adapt and change the pH of the surrounding environment, thereby inhibiting the growth of oral pathogens.[66] The antimicrobial substances secreted by *L. reuteri* are reuterin and

reutericycline.[67] Reuterin and reutericycline are broad-spectrum antimicrobial agents that are effective against Gram-positive and -negative bacteria, fungi, and protozoa by inhibiting microbial DNA synthesis.[35,47]

Many dental and oral health care products used daily now include probiotics. The use of probiotics is increasing due to their advantages over chemical agents, namely reducing the risk of antibiotic resistance.[68] Probiotics work by modulating the immune system, producing antimicrobial substances, and inhibiting certain pathogenic organisms by interfering with adhesion, colonization, and biofilm formation. They inhibit the growth of pathogens via the production of various substances, such as lactic acid and acetic acid, which penetrate the bacterial cell membrane and lower the cytoplasmic pH of pathogenic bacteria. Hydrogen peroxide and bacteriocin can destroy the cell membrane of pathogenic bacteria and inhibit the synthesis of pathogenic DNA.[32,33,35,69] Based on the results of this study, 2 weeks of daily consumption of the probiotic *L. reuteri* affected the process of biofilm formation by downregulating the expression of *BCR1*, *ACE2, fadA, aid1, gelE,* and *atlA g*enes, which function as adherent regulators and regulators of hyphae formation in biofilm formation. Many previous studies demonstrated that the addition of probiotics to dental and oral health care products. Probiotic can reduce pathogenic microorganisms in plaque samples from patients using fixed orthodontic appliances.[47,68,70–72] Therefore, it can be stated that the probiotic *L. reuteri* has good ability as an additional treatment for dental and oral health in patients using fixed orthodontic appliances.

The limitation of this study is the small sample size and the fact that the sampling had to be done during COVID-19 pandemic. Although the sample size is small, the result of this study can still provide concise and significant result. In the future, there should be larger sample size for conducting this research so the results can more accurately represent the actual population. Daily consumption of probiotic lozenges duration can also be increased for more precise and accurate results. On the other hand, consumption of probiotic lozenges research can also be conducted for other aspect of oral health diseases to promote the functionality and health-inducing aspect of probiotic lozenges, such as antiinflammation.

Conclusion

Within the limitation of this study, it can be concluded that the probiotic *L. reuteri* influences the expression of *BCR1*, *ACE2*, *fadA, aid1, gelE,* and *atlA* genes in biofilm formation. By reducing the expression of those genes, the probiotic *L. reuteri* can reduce biofilm formation, such as dental plaque, in patients using fixed orthodontic appliances.

Clinical Significance

Consumption of probiotic lozenges were confirmed to reduce bacterial and fungal biofilm, as proven by the reduction of virulence gene expression, hence, helping increasing oral health of consumer. The results of this study help clinicians provide probiotic lozenges for patients to promote and maintain their oral health.

References

1. Sihotang BM, Panjaitan M, et al. Perbedaan oral hygiene pemakaian pesawat ortodonti cekat dan pesawat ortodonti lepasan pada pasien klinik spesialis RS Herna. J Prima Medika Sains 2015;2(1):1–5. DOI: 10.34012/jpms.v2i1.840.
2. Gkantidis N, Christou P, Topouzelis N. The orthodontic-periodontic interrelationship in integrated treatment challenges: A systematic review. J Oral Rehabil 2010;37(5):377–390. DOI: 10.1111/j.1365-2842.2010.02068.x.
3. Oktaviani V, Santoso O. Perbedaan Indeks Higiene Oral dan pH Plak Kelompok Pemakai dan Bukan Pemakai Pesawat Ortodonti Cekat 2016;5(1):8.
4. Bowen WH, Burne RA, Wu H, et al. Oral biofilms: pathogens, matrix and polymicrobial interactions in microenvironments. Trends Microbiol 2018;26(3):229–242. DOI: 10.1016/j.tim.2017.09.008.
5. Huang R, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence 2011;2(5):435–444. DOI: 10.4161/viru.2.5.16140.
6. Verrusio C, Iorio-Siciliano V, Blasi A, et al. The effect of orthodontic treatment on periodontal tissue inflammation: A systematic review. Quintessence Int 2018;49(1):69–77. DOI: 10.3290/j.qi.a39225.
7. Perkowski K, Baltaza W, Conn DB, et al. Examination of oral biofilm microbiota in patients using fixed orthodontic appliances in order to prevent risk factors for health complications. Ann Agric Environ Med 2019;26(2):231–235. DOI: 10.26444/aaem/105797.
8. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. Curr Opin Microbiol 2015;23:141–147. DOI: 10.1016/j.mib.2014.11.013.
9. Rickard AH, Gilbert P, High NJ, et al. Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol 2003;11(2):94–100. DOI: 10.1016/s0966-842x(02)00034-3.
10. Thurnheer T, Karygianni L, Flury M, et al. Fusobacterium species and subspecies differentially affect the composition and architecture of supra- and subgingival biofilms models. Front Microbiol 2019;10:1716. DOI: 10.3389/fmicb.2019.01716.
11. Chew J, Zilm PS, Fuss JM, et al. A proteomic investigation of Fusobacterium nucleatum alkaline-induced biofilms. BMC Microbiol 2012;12(1):189. DOI: 10.1186/1471-2180-12-189.
12. Kaplan CW, Lux R, Haake SK, et al. The Fusobacterium nucleatum outer membrane protein RadD is an arginine-inhibitable adhesin required for inter-species adherence and the structured architecture of multi-species biofilm. Mol Microbiol 2009;71(1):35–47. DOI: 10.1111/j.1365-2958.2008.06503.x.
13. Cochrane K, Robinson AV, Holt RA, et al. A survey of Fusobacterium nucleatum genes modulated by host cell infection. Microb Genom 2020;6(2):e000300. DOI: 10.1099/mgen.0.000300.
14. Kaplan A, Kaplan CW, He X, et al. Characterization of aid1, a novel gene involved in Fusobacterium nucleatum interspecies interactions. Microb Ecol 2014;68(2):379–387. DOI: 10.1007/s00248-014-0400-y.
15. Liu P, Liu Y, Wang J, et al. Detection of Fusobacterium nucleatum and fadA adhesin gene in patients with orthodontic gingivitis and non-orthodontic periodontal inflammation. PLOS One 2014;9(1):e85280. DOI: 10.1371/journal.pone.0085280.
16. Brennan CA, Garrett WS. Fusobacterium nucleatum – symbiont, opportunist and oncobacterium. Nat Rev Microbiol 2019;17(3):156–166. DOI: 10.1038/s41579-018-0129-6.
17. Wu T, Cen L, Kaplan C, et al. Cellular components mediating coadherence of Candida albicans and Fusobacterium nucleatum. J Dent Res 2015;94(10):1432–1438. DOI: 10.1177/0022034515593706.
18. Wang Q-Q, Zhang C-F, Chu C-H, et al. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. Int J Oral Sci 2012;4(1):19–23. DOI: 10.1038/ijos.2012.17.
19. Najafi K, Gholizadeh P, Tanomand A, et al. Oral cavity infection by Enterococcus faecalis: Virulence factors and pathogenesis. Rev Med Microbiol 2019;30(2). DOI: 10.1097/MRM.0000000000000168.
20. Ch'ng J-H, Chong KKL, Lam LN, et al. Biofilm-associated infection by enterococci. Nat Rev Microbiol 2019;17(2):82–94. DOI: 10.1038/s41579-018-0107-z.
21. Anderson AC, Jonas D, Huber I, et al. Enterococcus faecalis from food, clinical specimens, and oral sites: prevalence of virulence factors in association with biofilm formation. Front Microbiol 2016;6:1534. DOI: 10.3389/fmicb.2015.01534.
22. Stinemetz EK, Gao P, Pinkston KL, et al. Processing of the major autolysin of E. faecalis, AtlA, by the zinc-metalloprotease, GelE,

impacts AtlA septal localization and cell separation. PLoS One 2017;12(10):e0186706.
23. Guiton PS, Hung CS, Kline KA, et al. Contribution of autolysin and sortase A during Enterococcus faecalis DNA-dependent biofilm development. Infect Immun 2009;77(9):3626–3638. DOI: 10.1128/IAI.00219-09.
24. Paganelli FL, Willems RJL, Jansen P, et al. Enterococcus faecium biofilm formation: identification of major autolysin AtlAEfm, associated Acm surface localization, and AtlAEfm-independent extracellular DNA release. mBio 2013;4(2):e00154. DOI: 10.1128/mBio.00154-13.
25. Putri MH, Herijulianti E. Ilmu Pencegahan Penyakit Jaringan Keras dan Jaringan Pendukung Gigi | OPAC Integrasi | Online Public Access Catalog | Universitas Gadjah Mada. RGC; 2010.
26. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: A persistent public health problem. Clin Microbiol Rev 2007;20(1):133–163. DOI: 10.1128/CMR.00029-06.
27. Garcia MC, Lee JT, Ramsook CB, et al. A role for amyloid in cell aggregation and biofilm formation. PLoS One 2011;6(3):e17632. DOI: 10.1371/journal.pone.0017632.
28. Nobile CJ, Mitchell AP. Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Curr Biol 2005;15(12):1150–1155. DOI: 10.1016/j.cub.2005.05.047.
29. Mulhern SM, Logue ME, Butler G. Candida albicans transcription factor Ace2 regulates metabolism and is required for filamentation in hypoxic conditions. Eukaryot Cell 2006;5(12):2001–2013. DOI: 10.1128/EC.00155-06.
30. Saputo S, Kumar A, Krysan DJ. Efg1 directly regulates ACE2 expression to mediate cross talk between the cAMP/PKA and RAM pathways during Candida albicans morphogenesis. Eukaryot Cell 2014;13(9):1169–1180. DOI: 10.1128/EC.00148-14.
31. Widyarman AS, Bachtiar EW, Bachtiar BM, et al. Inhibitory effect of probiotic lactobacilli against Streptococcus mutans and Porphyromonas gingivalis biofilms. Sci Dental J 2019;3(2):50. DOI: 10.4103/SDJ.SDJ_8_19.
32. Widyarman AS, Ramajayanti T, Theodorea CF. Indonesian strain of probiotic reduces the initial biofilm colonization. The Open Dent J 2020;14(1). DOI: 10.2174/1874210602014010544.
33. Widyarman AS, Theodorea CF. Novel indigenous probiotic Lactobacillus reuteri strain produces anti-biofilm reuterin against pathogenic periodontal bacteria. Eur J Dent 2021;16(1):96–101. DOI: 10.1055/s-0041-1731591.
34. Mu Q, Tavella VJ, Luo XM. Role of Lactobacillus reuteri in human health and diseases. Front Microbiol 2018;9:757. DOI: 10.3389/fmicb.2018.00757.
35. Sari Widyarman A, Kiky Elysia Lazaroni N. Persistent endodontics pathogens biofilm inhibited by Lactobacillus reuteri Indonesian strain. J Dent Indones 2019;26(3). Available from: https://scholarhub.ui.ac.id/jdi/vol26/iss3/7/.
36. Collado MC, Meriluoto J, Salminen S. Adhesion and aggregation properties of probiotic and pathogen strains. Eur Food Res Technol 2008;226(5):1065–1073. DOI: 10.1007/s00217-007-0632-x.
37. Julious SA. Sample size of 12 per group rule of thumb for a pilot study. Pharm Stat 2005;4:287–291. DOI: 10.1002/pst.185.
38. Johanson GA, Brooks GP. Initial scale development: Sample size for pilot studies. Educ Psychol Meas 2010;70:394–400. DOI: 10.1177/0013164409355692.
39. Nikoomanesh F, Roudbarmohammadi S, Roudbary M, et al. Investigation of bcr1 gene expression in Candida albicans isolates by RT-PCR technique and its impact on biofilm formation. iem 2016;2(1):22–24. DOI: 10.7508/iem.2016.01.007.
40. Ranjith K, Kalyana Chakravarthy S, Adicherla H, et al. Temporal expression of genes in biofilm-forming ocular *Candida albicans* isolated from patients with keratitis and orbital cellulitis. Invest Ophthalmol Vis Sci 2018;59(1):528–538. DOI: 10.1167/iovs.17-22933.
41. Nailis H, Coenye T, Van Nieuwerburgh F, et al. Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. BMC Mol Biol 2006;7:25. DOI: 10.1186/1471-2199-7-25.
42. Vos M, Quince C, Pijl AS, et al. A Comparison of rpoB and 16S rRNA as markers in pyrosequencing studies of bacterial diversity. PLoS One 2012;7(2):e30600. DOI: 10.1371/journal.pone.0030600.
43. Wang L, Dong M, Zheng J, et al. Relationship of biofilm formation and gelE gene expression in Enterococcus faecalis recovered from root canals in patients requiring endodontic retreatment. J Endod 2011;37(5):631–636. DOI: 10.1016/j.joen.2011.02.006.
44. Zheng J-X, Wu Y, Lin Z-W, et al. Characteristics of and virulence factors associated with biofilm formation in clinical Enterococcus faecalis isolates in China. Front Microbiol 2017;8:2338. DOI: 10.3389/fmicb.2017.02338.
45. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. J Oral Microbiol 2011;3. DOI: 10.3402/jom.v3i0.5771.
46. Khanpayeh E, Jafari AA, Tabatabaei Z. Comparison of salivary Candida profile in patients with fixed and removable orthodontic appliances therapy. Iran J Microbiol 2014;6(4):263–268. PMID: 25802710.
47. Widyarman AS, Hartono V, Marjani LI, et al. Lactobacillus reuteri containing probiotic Lozenges consumption reduces Streptococcus mutans, Streptococcus sobrinus, Porphyromonas gingivalis, and Aggregatibacter actinomycetemcomitans in orthodontic patients. J Int Dent Med Res 2018;11(2):628–633.
48. Restrepo M, Bussaneli DG, Jeremias F, et al. Control of white spot lesions with use of fluoride varnish or chlorhexidine gel during orthodontic treatment A randomized clinical trial. J Clin Pediatr Dent 2016;40(4):274–280. DOI: 10.17796/1053-4628-40.4.274.
49. Lu H, Tang H, Zhou T, et al. Assessment of the periodontal health status in patients undergoing orthodontic treatment with fixed appliances and Invisalign system. Medicine (Baltimore) 2018;97(13):e0248. DOI: 10.1097/MD.0000000000010248.
50. Fitriasari N, Wahyuni IS. POTENSI PROBIOTIK DALAM TATALAKSANA ORAL CANDIDIASIS: Ulasan Sistematik. Odonto : Dent J 2021;8(1):34–44. DOI: 10.30659/odj.8.1.34-44.
51. Guo L, Feng Y, Guo H-G, et al. Consequences of orthodontic treatment in malocclusion patients: clinical and microbial effects in adults and children. BMC Oral Health 2016;16(1):112. DOI: 10.1186/s12903-016-0308-7.
52. Alqerban A. Efficacy of antimicrobial photodynamic and photobiomodulation therapy against Treponema denticola, fusobacterium nucleatum and human beta defensin-2 levels in patients with gingivitis undergoing fixed orthodontic treatment: a clinic-laboratory study. Photodiagnosis Photodyn Ther 2020;29:101659.
53. Willett JLE, Ji MM, Dunny GM. Exploiting biofilm phenotypes for functional characterization of hypothetical genes in Enterococcus faecalis. NPJ Biofilms Microbiomes 2019;5(1):23. DOI: 10.1038/s41522-019-0099-0.
54. Sinaga E. Regulasi Ekspresi Gen. Regulasi Ekspresi Gen 2011;1–24.
55. Van Peer G, Mestdagh P, Vandesompele J. Accurate RT-qPCR gene expression analysis on cell culture lysates. Sci Rep 2012;2:222. DOI: 10.1038/srep00222.
56. Shemesh M, Tam A, Steinberg D. Expression of biofilm-associated genes of Streptococcus mutans in response to glucose and sucrose. J Med Microbiol 2007;56(11):1528–1535. DOI: 10.1099/jmm.0.47146-0.
57. Fanning S, Xu W, Solis N, et al. Divergent targets of Candida albicans biofilm regulator Bcr1 in vitro and in vivo. Eukaryot Cell 2012;11(7):896–904. DOI: 10.1128/EC.00103-12.
58. Matsubara VH, Bandara HMHN, Mayer MPA, et al. Probiotics as antifungals in mucosal candidiasis. Clin Infect Dis 2016;62(9):1143–1153. DOI: 10.1093/cid/ciw038.
59. Li Q, Tan L, Wang H, et al. Fusobacterium nucleatum interaction with Pseudomonas aeruginosa induces biofilm-associated antibiotic tolerance via Fusobacterium adhesin A. ACS Infect Dis 2020;6(7):1686–1696. DOI: 10.1021/acsinfecdis.9b00402.
60. Kaplan A. Identification and characterization of a novel lipoprotein in Fusobacterium nucleatum. UCLA; 2015 Available from: https://escholarship.org/uc/item/3r90n33c.
61. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402–408.

62. Barzegari A, Kheyrolahzadeh K, Hosseiniyan Khatibi SM, et al. The battle of probiotics and their derivatives against biofilms. Infect Drug Resist 2020;13:659–672. DOI: 10.2147/IDR.S232982.
63. Guneser MB, Eldeniz AU. The effect of gelatinase production of Enterococcus faecalis on adhesion to dentin after irrigation with various endodontic irrigants. Acta Biomater Odontol Scand 2016;2(1):144–149. DOI: 10.1080/23337931.2016.1256212.
64. Hashem YA, Amin HM, Essam TM, et al. Biofilm formation in enterococci: Genotype-phenotype correlations and inhibition by vancomycin. Sci Rep 2017;7(1):5733. DOI: 10.1038/s41598-017-05901-0.
65. Ali L, Goraya MU, Arafat Y, et al. Molecular mechanism of quorum-sensing in Enterococcus faecalis: its role in virulence and therapeutic approaches. Int J Mol Sci 2017;18(5):960. DOI: 10.3390/ijms18050960.
66. Bonifait L, Chandad F, Grenier D. Probiotics for oral health: myth or reality? J Can Dent Assoc 2009;75(8):585–590. PMID: 19840501
67. Urbańska M, Szajewska H. The efficacy of Lactobacillus reuteri DSM 17938 in infants and children: A review of the current evidence. Eur J Pediatr 2014;173(10):1327–1337. DOI: 10.1007/s00431-014-2328-0.
68. Benic GZ, Farella M, Morgan XC, et al. Oral probiotics reduce halitosis in patients wearing orthodontic braces: A randomized, triple-blind, placebo-controlled trial. J Breath Res 2019;13(3):036010. DOI: 10.1088/1752-7163/ab1c81.
69. Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S, et al. Probiotic mechanisms of action. Ann Nutr Metab 2012;61(2):160–174. DOI: 10.1159/000342079.
70. Widyarman AS, Yunita ST, Prasetyadi T. Consumption of yogurt containing probiotic *Bifidobacterium lactis* reduces *Streptococcus mutans* in orthodontic patients. Sci Dent J2018;2(1):19–25. DOI: 10.26912/sdj.v2i1.1913.
71. Alp S, Baka ZM. Effects of probiotics on salivary *Streptecoccus mutans* and Lactobacillus levels in orthodontic patients. Am J Orthod Dentofacial Orthop 2018;154(4):517–523. DOI: 10.1016/j.ajodo.2018.01.010.
72. Goyal N, Shamanna PU, Varughese ST, et al. Effects of amine fluoride and probiotic mouthwash on levels of *Porphyromonas gingivalis* in orthodontic patients: a randomized controlled trial. J Indian Soc Periodontol 2019;23(4):339–344. DOI: 10.4103/jisp.jisp_551_18.