Lactobacillus reuteri containing Probiotic Lozenges Consumption Reduces *Streptococcus mutans, Streptococcus sobrinus, Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans* in Orthodontic Patients

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Abstract

Objective of this study is to investigate the effect of daily consumption of *Lactobacillus reuteri* ATCC 55730 containing probiotic lozenges on salivary *Streptococcus mutans* serotype c, *Streptococcus sobrinus, Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans* number, in patients undergoing orthodontic treatment with fixed appliance.

Saliva subject collected before and two weeks after daily probiotic consumption. *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis* ATCC 33277, and *A. actinomycetemcomitans* ATCC 29522 were cultured in BHI-broth (anaerobic-condition). After 48-h incubation, the number of colonies on each dilution plate were used to extrapolate a standard curve. Bacterial DNA were extracted using heat-shock method. Subsequently, quantitative Real-Time PCR method was applied to analyse the number of *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans* in saliva. Total number of DNA target were identified using SYBR Green (Applied Biosystem) and 16S rRNA gene specific primers for each bacterium tested.

The research results showed that there was significant difference in the average scores of *S. mutans* serotype, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans* before probiotic consumption compared to two weeks after probiotic consumption. The results show that probiotic L. reuteri ATCC-55730 brought a statistically significant reduction of *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans* after two weeks consumption of probiotic in fixed orthodontic patients ($P< 0.05$).

It is concluded that *L. reuteri* ATCC 55730-containing probiotic lozenges consumption reduces the number of *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans* in saliva subjects during fixed orthodontic appliances.

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Introduction

Fixed orthodontic appliances is a risk factor in developing dental caries and periodontal disease. It is caused by the presence of brackets, bands, and arch wire that facilitate accumulation and retention of dental plaque, as well as complicate oral cleansing[1]. *Streptococcus mutans* is a facultative anaerobic Gram-positive oral bacterium that has an important role in developing dental caries and is one of several species belong to the mutan streptococci. Species within the mutans streptococci are differentiated by their serotypes, such as *S. mutans* (serotype c, e, f, and k) and *S. sobrinus* (serotype d and g)[2–4]. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are an anaerobic Gram-negative oral bacteria that involved in the pathogenesis of periodontitis, an inflammatory disease that characterized by the destruction of connective tissue and dental bone support following an inflammatory host response secondary to infection by periodontal bacteria[5,6].

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An expert in the organization of the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) states that the term of probiotic is used to describe a living microorganisms which, when administered in adequate amounts, confer a health benefit on the host [7]. There are several strains that can be classified as probiotics, and they commonly belong to the Lactobacillus, Bifidobacterium and Streptococci genera[7,8]. *Lactobacillus reuteri* ATCC 55730 is one of the probiotic bacteria that has been identified and is categorized as one of the lactic acid-producing bacteria (LAB)[9]. *L. reuteri* ATCC 55730 can produce antimicrobial substances (*reuterin* and *reutericyclin*) which are active against Gram-positive and Gram-negative bacteria, yeast, fungi, or parasites[10]. Thus, the authors conducted further research to study the probiotic *L. reuteri* ATCC 55730 effect to *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans.* on saliva in patients undergoing orthodontic treatment with fixed appliance.

Methodology

Subjects were chosen from students of Faculty of Dentistry, Trisakti University, undergoing orthodontic treatment with fixed appliance, with following criteria: male of female age of 20—30; undergoing orthodontic treatment with fixed appliance for the last 6 months; no consumption of probiotics (min. 3 months prior); no history of systemic disease i.e. diabetics, chronic kidney failure, etc. that can affect saliva substance; no consumption of medicine i.e. anticonvulsant, etc. that can affect saliva substance.

Consented to be research participant. This study has obtained the permission from ethics committee with number: 224/KE/FKG/12/2015

Number of samples were calculated from following calculation,

$$n = \frac{Z^2.N.p.q}{d^2(N-1)+Z^2.p.q} = \frac{1{,}96^2.8.0{,}5.0{,}5}{0{,}1^2(8-1)+1{,}96^2.0{,}5.0{,}5} \approx 8$$

n: minimum number of samples
N: number of population
p: population target proportion (0,5)
q: proportion without attribute (1-p) = 0,5
Z: normal standard deviation for 1,96 with CI of 95%
d: level of significance of 90% or 0,1

Saliva (n=8, mean ages 21 years) were collected before treatment and after two weeks of daily treatment of the probiotic lozenges administered ($2x10^8$ CFU/tablet) after breakfast and tooth brushing by using a sterile funnel to transfer the saliva into a 15-mL micro-centrifuge tube until 2 mL saliva obtained in each tube. The collected saliva samples were transferred to the laboratory and stored in freezer at -20 °C until use.

Microbial strains and growth conditions

S. mutans serotype c and *S. sobrinus* were cultured in Brain Heart Infusion broth (Thermo Scientific, Waltham, MA, USA) at 37°C for 48 hours, in anaerobic condition (10% CO_2, 10% H,80% N_2). *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* ATCC 29522 were cultured in BHI broth and incubated in a GasPak jar system (Becton Dickinson, Franklin Lakes, NJ, USA). The standard plate count method was used to determine the bacterial enumeration by serial dilution technique. After 48 hours incubation, the number of colonies on each dilution plate used to extrapolate a standard curve.

Bacterial DNA Extraction

Bacterial DNA from saliva sample and cultured bacteria for standard curve were extracted using the heat-shock method. The samples were centrifuged at 4500 xg for 15 min at 4°C and pellets were washed with Phosphate Buffer Saline (PBS). 100 µL of each bacterial cell suspension (10^8 cells/mL) of *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans* in microtubes were centrifuge at 10.000 xg for 10 min at 4°C, then incubated at 99°C in a boiling water for 20 min and homogenized by vortex for 10 second and immediately the tube was frozen on ice box (0°C) for 10 min. Subsequently, centrifugation was done at 10.000 x g for 2 min and supernatant was pipetted into 1.5 mL micro-centrifuge tubes. The suspension containing DNA sample was stored at -20 °C.

Quantitative Real-Time PCR

A quantification using Real-Time PCR with SYBR Green chemistry fluorescence

(Applied Biosystems, USA) was applied to analyze the number of *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans* from saliva sample. The total number of DNA target were identified using *GtfB* gene specific primers for *S. mutans* serotype c[11]; *GtfT* gene specific primers for *S. sobrinus*[12]; 16S rRNA gene specific primers for *P. gingivalis*[11]; 16S rRNA gene specific primers for *A. actinomycetemcomitans* [13] (Table 1).

Primers	Sequence (5'-3')
S. mutans serotype c (*gtfB gene*) Forward	GCC TAC AGC TCA GAG ATG CTA TTC T
S. mutans serotype c (*gtfB gene*) Reverse	GCC ATA CAC CAC TCA TGA ATT GA
S. sobrinus serotype d (*gtfT gene*) Forward	ACT ACA CTT TCG GGT GGC TTG G
S. sobrinus serotype d (*gtfT gene*) Reverse	CAG TAT AAG CGC CAG TTT CAT C
P. gingivalis (16S rRNA) Forward	TGC AAC TTG CCT TAC AGA GGG
P. gingivalis (16S rRNA) Reverse	ACT CGT ATC GCC CGT TAT TC
A. actinomycetemcomitans (16S sRNA) Forward	CTT ACC TAC TCT TGA CAT CCG AA
A. actinomycetemcomitans (16S sRNA) Reverse	ATG CAG CAC CTG TCT CAA AGC

Table 1. Primers used for the RT-PCR method [10-12]

The reaction mixture in 20 µL volume contained: 10 µL SYBRGreen reagent, 1 µL of forward primers, 1 µL of forward primers, 2 µL template DNA and 6 µL nuclease free water (NFW). Amplifications were done with the following temperature profiles. Initial template denaturation step at 95 °C for 10 minutes (1 cycle), followed by 40 cycles of 94 °C for 15 seconds and annealing at 57-62 °C for 1 min, 95 °C for 15 seconds.[14] The RT-PCR was performed in triplicate. Quantitation was done using standard curves made from known concentrations of DNA containing the respective amplicon for each set of primers

Statistical analysis

The obtained results were statistically analyzed by paired T-test to reveal significant differences of bacterial number before and after probiotic consumption. Level of significance was set at $P<0.05$. Shapiro Wilk test was used to test for normality and Levene's test was used to test for homogeneity of variance previously. Statistical calculations were performed with SPSS Statistics for Windows software version 20 (IBM, USA). This study has obtained the permission from ethics committee with number: 224/KE/FKG/12/2015

Results

The number of salivary *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans* for pre- and post-probiotic consumption in all subjects are shown in figure 1-4.

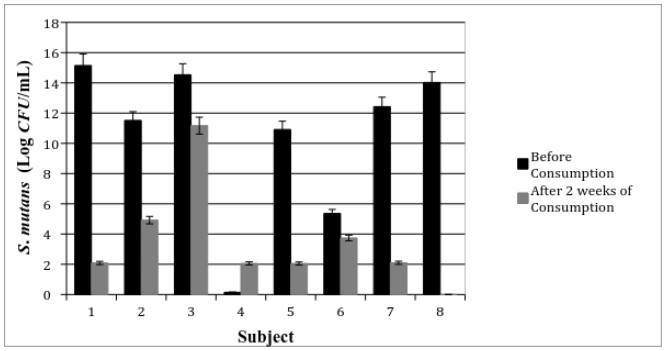

Figure 1. Reduction number of *Streptococcus mutans* serotype c in saliva before and two weeks after probiotic consumption (Log CFU/mL)

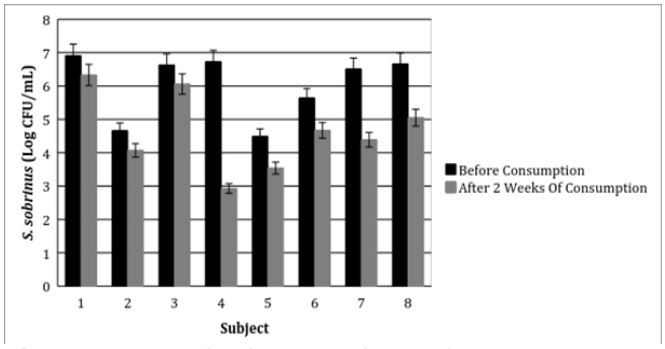

Figure 2. Reduction number of *Streptococcus sobrinus* serotype d in saliva before and two weeks after probiotic consumption (Log CFU/mL)

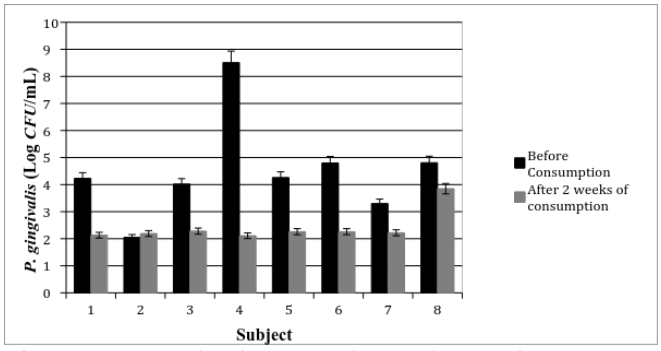

Figure 3. Reduction number of *Porphyromonas gingivalis* in saliva before and two weeks after probiotic consumption (Log CFU/mL)

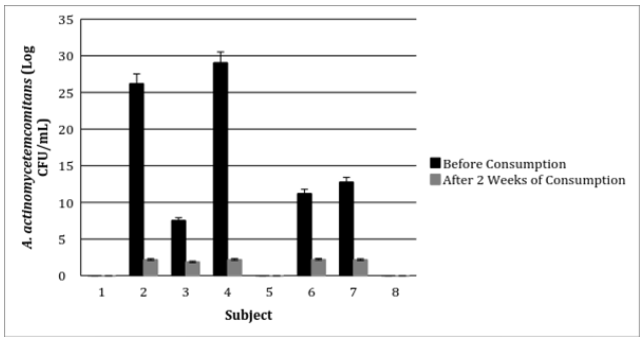

Figure 4. Reduction number of *Aggregatibacter actinomycetemcomitans* in saliva before and two weeks after probiotic consumption (Log CFU/mL)

The figures show reduction number of salivary *S. sobrinus* in all subjects, while the total number of *S. mutans* serotype c and *P. gingivalis* in the saliva of seven subjects were decreased, and reduction number of *A. actinomycetemcomitans* in five subjects.

The results showed that there were significant difference in the average scores of *S. mutans* serotype c (14.37±3.32) log CFU/mL, *S. sobrinus* serotype d (6.028±0.137) log CFU/mL, *P. gingivalis* (7.61±1.85) log CFU/mL, and *A. actinomycetemcomitans* (10.85±1.77) log CFU/mL before probiotic consumption compared to average scores of *S. mutans* serotype c (10.27±3.48) log CFU/mL, *S. sobrinus* (4.63±0.037) log CFU/mL, *P. gingivalis* (2.21±0.61) log CFU/mL, and *A. actinomycetemcomitans* (1.61±0.06) log CFU/mL, two weeks after probiotic consumption (Figure 1-4). The results show that probiotic *L. reuteri* ATCC-55730 brought a statistically significant reduction of *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans* after two weeks consumption of probiotic in fixed orthodontic patients ($p< 0.05$) respectively.

Discussion

Number of colonies counted in the saliva presents the number of bacterial colonies on the surface of the hard tissue and soft tissues in the oral cavity of orthodontic patients. Therefore, this research used saliva from the subjects as a sample. The consideration of choosing patients undergoing fixed orthodontic treatment who using orthodontic appliance such as brackets, bands, and arch wire enabled to induce changes in buffer capacity, salivary flow rate, acidity (pH), causing plaque accumulation that lead to an increase in both caries and periodontal disease prevalence[15–17].

Results showed that seven out of eight subjects experienced a decrease in the number of *S. mutans* serotype c and eight subjects showed a decrease in the number of *S. sobrinus* significantly after 2 weeks of consuming probiotics. Nikawa *et al*, revealed that daily consumption of *L. reuteri*-containing yogurt showed a reduction in the number of *S. mutans* and *S. sobrinus* in human oral cavity [18,19].

It was stated because *L. reuteri* is capable of producing a broad-spectrum antimicrobial

agent called reuterin (3-hydroxy-propionaldehyde) was able to show great antimicrobial activity against *S. mutans*[20–22].

L. reuteri is known for its secretion of 2 bacteriocins, reuterin and reutericyclin, that inhibit the growth of a wide variety of pathogens by acting as a competitive inhibitor for an enzyme needed in DNA synthesis[23–26]. *L. reuteri* also has a strong capacity to adhere to host tissues, thereby competing with pathogenic bacteria[27]. Based on study, *Baca-Castanon* et al proved that *L. reuteri* has an antimicrobial activity to inhibit the growth of pathogenic bacteria (*S. mutans*, *Streptococcus gordonii*, and *Tannerella forsythia*) using Kirby Bauer method. Therefore, *L. reuteri* could be used as an alternative to prevent or treat oral cavity diseases[28].

L. reuteri strongly inhibit or suppress the growth of a major periodontal pathogens (*P. gingivalis*) in biofilm-competition assay and reduce the growth rate of *mutans streptococci*[29–32]. *Koll-Klais,* et al revealed that *Lactobacillus* strains can suppress the growth of periodontal pathogens such as *P. gingivalis*, *A. actinomycetemcomitas*, and *Prevotella intermedia*[33]. Another study showed that consuming probiotic yogurt containing *Bifidobacterium lactis* BB-12 reduced the number of *S. mutans* in the saliva of subjects during fixed orthodontic treatment.[14] In this study, results showed that seven out of eight subjects experienced a decrease in the number of *P. gingivalis* and five subjects showed a decrease in the number of *A. actinomycetemcomitans* significantly after 2 weeks of consuming probiotics.

L. reuteri and *L. brevis* probiotics can reduce swelling and gingival bleeding[34]. Other clinical trials have also shown an inhibition of pathogen colonization in the periodontal pocket and also reduction of bleeding on probing, probing depth and the prevalence of gingival inflammation severity as well as Plaque Index (PI) in adults after routine use of probiotic tablets [35,36].

Furthermore, Krasse et al has been proven that chewing *L. reuteri*-containing gum was effective in reducing both gingivitis and plaque in patient with moderate to severe gingivitis[37–39].

In this study nearly all the subjects showed a reduction in the number of *S. mutans* serotype c, *S. sobrinus*, *P. gingivalis*, and *A. actinomycetemcomitans* in saliva, however there is one subject that showed an increasing number of *S. mutans* serotype c, *P. gingivalis*, while *A. actinomycetemcomitans* were undetectable in three subjects. It was possible because of lack of subject's cooperation and poor oral hygiene that facilitate colonization of those pathogenic bacteria[40–42].

Conclusion

Based on these results, it is concluded that two weeks of daily consumption of L. reuteri ATCC 55730-containing probiotic lozenges enables the reduction the number of salivary S. mutans serotype c, S. sobrinus, P. gingivalis, and A. actinomycetemcomitans in fixed orthodontic patients. Future studies are needed to explore this effect.

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