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Original Research

In-Vitro Inhibitory Efficacy of 3 Types of Probiotics on the Growth of *Aggregatibacter actinomycetemcomitans* Bacteria

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Abstract

Background: Amongst the specific plaque pathogen *Aggregatibacter actinomycetemcomitans* (Aa) ATCC 43718 serotype b is one of the highly virulent bacteria that causes periodontitis. Probiotic therapy is a treatment in which the lactic acid bacteria in are utilized to impede the colonization and growth of the pathogenic bacteria to prevent the further formation of dental plaque. **Objective**: The present research aimed to evaluate inhibiting effect of purified bacteria from various commercially available yogurt product containing bacteria named (*Lactobacillus casei* strain Shirota; *Lactobacillus bulgaricus* and *Streptococcus thermophilus*; *Lactobacillus reuteri* Prodentis) on the growth of Aa. **Methods**: The research made use of the diffusion method by fixing Aa on BHIB (brain heart infusion broth) medium, incubated at 37 °C and 24 hours later planted on MHA (Mueller-Hinton agar) media. Aa were divided into four subgroups each with a paper disk; group 1 consists of untreated bacteria (i.e., control group), group 2 with purified bacteria from Yakult 0.5 μL, group 3 with purified bacteria from Cimory Yogurt Drink 0.5 μL and group 4 with purified bacteria from BioGaia Prodentis 0.5 μL. All commercially available yogurt were treated to get the purified probiotic. Additionally, it was incubated for 24 hours at 37 °C and later the inhibition zone diameter was observed. **Results**: In the research, it was found that the average impeding ability, so-called inhibition zone, in group 1 indicated 0 mm, group 2 indicated 12.70 mm, group 3 indicated 16.60 mm and group 4 indicated 19.60 mm. The statistical test outcomes showed a significance of 0.000 ($p < 0.05$). **Conclusions**: The purified bacteria from three probiotics indeed inhibit the growth of the Aa bacteria and a substantial difference in the diameter of the inhibition zone were found among the three probiotics.

Keywords: *Lactobacillus casei* strain Shirota; *Lactobacillus bulgaricus* and *Streptococcus thermophilus*; *Lactobacillus reuteri* Prodentis; *Aggregatibacter actinomycetemcomitans*; probiotics; healthy lifestyle

1. Introduction

Periodontitis is an oral disease mainly affecting supporting tissue (gingiva, periodontal ligament and alveolar bone) around the tooth with high frequency of occurrence, whereas the percentage of such cases in Indonesia goes as high as 74.1% [1–4]. *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, and *Porphyromonas gingivalis* were identified as specific periodontal pathogens in periodontal disease. *A. actinomycetemcomitans* strongly associated with the aggressive forms of the disease [5]. The major cause of periodontitis is the existence of pathogenic bacteria which accumulate and later colonize in the dental plaque [6]. Attempts in controlling the amount of plaque around the teeth is a main stay of treatment of periodontal disease [7].

Various attempts are made to break the chain of bacterial adherence in plaque matrix. Probiotics are live microorganisms that are able to provide advantageous impacts on the health of their hosts when consumed in adequate quantities [8–10]. Many advantages can be found in probiotics, including helping the immune response, increasing resistance to pathogenic bacteria, reducing harmful bacteria, and maintaining the balance of healthy microbes in the body. Several studies have shown that probiotics can impede the formation of plaque which is a predisposing factor for the diseases in the oral cavity such as; caries, halitosis and periodontal disease [11–13]. Bacteria in probiotics helps to impede the adhesion and invasion of pathogenic bacteria [14,15].

This research is aimed at observing the benefits of probiotics for the oral cavity, mainly in the treatment of periodontal disease. *Lactobacillus casei* strain Shirota bacteria play a beneficial role in reducing gingival inflammation and improving periodontal health due to the fact that this probiotic bacteria can actually cut down the number of *A. actinomycetemcomitans* bacteria in dental biofilm [14,16].

Lactobacillus bulgaricus and *Streptococcus thermophilus* will produce organic acids, e.g., lactic acid, acetic acid, formic acid, hydrogen peroxide, diacetyl and bacteri-

ocin which all have antibacterial properties during the process of fermentation [17,18]. *L. bulgaricus* and *S. thermophilus* exhibits beneficial effects on periodontal tissue by their impeding effect on the growth of the *A. actinomycetemcomitans* bacteria [19]. The probiotic *Lactobacillus reuteri* Prodentis has the role of reducing gingival inflammation as well as decreasing the gingival bleeding and healing periodontal tissues after scaling and root planing [20,21]. *L. reuteri* produce reuterin which is an antimicrobial compound. Reuterin has been shown to be bioactive against bacteria, viruses, and fungi [22].

The investigating bacteria were divided into three experimental groups to observe and later to conclude their impeding strength against *A. actinomycetemcomitans*. Based on previous researches, probiotic *L. casei* strain Shirota, *L. bulgaricus* and *S. thermophilus*; *L. reuteri* Prodentis were capable of inhibiting the plaque bacteria causing periodontitis [14,23]. In this study researchers aimed to observe the antibacterial efficacy of commercially available probiotics in the market by inhibiting the growth of the *A. actinomycetemcomitans* that is one of the pathogens in the etiology of periodontitis. The null hypothesis to be tested is that the probiotics will not have inhibitory effect on the growth of *Aggregatibacter actinomycetemcomitans* (Aa).

2. Materials and Methods

2.1 The Required Sample Number

The minimum sample amount proper for further analysis was determined with Federer formula, in which t stood for the groups in the research treatment, and r stood for the number of replications in each group of petri dishes used in the research.

$(t–1) \times (r–1) \geq 15$
$(4–1) \times (r–1) \geq 15$
$3 \times (r–1) \geq 15$
$3r – 3 \geq 15$
$3r \geq 18$
$r \geq 6$

Thus, based on the calculation above, the least number of the required samples were 6 [24].

2.2 Sample Groups

The samples in the research were divided into 4 disk diffusion procedural groups. These groups consisted of, group 1 containing the pathogenic bacteria *A. actinomycetemcomitans* ATCC 43718 serotype b untreated with any probiotics (i.e., control group); group 2 was that which treated with *L. casei* strain Shirota bacteria; group 3 was that which was treated with *L. bulgaricus* and *S. thermophilus* bacteria; group 4 was that which was treated with *L. reuteri* Prodentis bacteria.

2.3 Bacteria Strain and Culture Conditions

Reference strain of *A. actinomycetemcomitans* ATCC 43718 serotype b. The bacteria were replaced in a reaction tube which has been provided with BHIB (brain heart infusion broth). The outcome of the culture was then placed in an anaerobic jar and incubated at 37 °C for 24 hours in anaerobic environment. Later the opacity was observed in order to be balanced with the standard of 0.5 McFarland (1.5×10^8 colony forming units (CFUs) mL^{-1}).

2.4 Probiotic Purification

The following is the method of probiotic purification.

2.4.1 Product A Probiotic Purification

Product A contained the *L. casei* strain Shirota in the Yakult drink manufactured by Perseroan Terbatas (PT), (Jakarta Selatan, Indonesia). Yakult Indonesia Persada. This probiotic was placed in a reactive tube which had been given the BHIB media. The reactive tube was then placed in an anaerobic jar incubated at 37 °C for 24 hours, which further incubated for 24 hours in anaerobic environment could be seen in Fig. 1. The resulted opacity was then observed with the 0.5 McFarland (1.5×10^8 CFUs mL^{-1}).

Fig. 1. Probiotic was placed in a reactive tube then placed in an anaerobic jar and incubated at 37 °C for 24 hours, which further incubated for 24 hours in anaerobic environment.

2.4.2 Product B Probiotic Purification

Product B contained the *L. bulgaricus* and *S. thermophilus* obtained from Cimory Yogurt Drink produced

by PT. Cisarua Mountain Dairy Tbk. This probiotic was placed in a reactive tube which had been given the BHIB media. The reactive tube was then placed in an anaerobic jar and incubated at 37 °C for 24 hours, which further incubated for 24 hours in anaerobic environment could be seen in Fig. 1. The resulted opacity was then observed with the 0.5 McFarland (1.5×10^8 CFUs mL^{-1}).

2.4.3 Product C Probiotic Purification

Product C contained the *L. reuteri* Prodentis contained within BioGaia Prodentis lozenges produced by BioGaia. This probiotic was placed in a reactive tube which had been given the BHIB media. The reactive tube was then placed in an anaerobic jar and incubated at 37 °C for 24 hours, which further incubated for 24 hours in anaerobic environment could be seen in Fig. 1. The resulted opacity was then observed with the 0.5 McFarland (1.5×10^8 CFUs mL^{-1}).

2.5 Inhibition Force Testing

A. actinomycetemcomitans ATCC 43718 serotype b bacterial colonies were taken from BHIB media with sterile cotton swabs. The bacteria were then planted on MHA using 3× cotton swabs at an angle of 60° could be seen in Fig. 2. The bacteria *A. actinomycetemcomitans* ATCC 43718 serotype b, were cultured with the method of spreading. After that paper disk was placed on MHA (Mueller-Hinton agar) could be seen in Fig. 3. Each purified probiotic was dropped onto the paper disk as much as 0.5 μL and incubated anaerobically in an incubator at 37 °C for 24 hours.

2.6 Inhibition Zone Measuring

The diameter measurement of the inhibition zone in each testing group was conducted with a Vernier caliper in millimeters could be seen in Fig. 4. The inhibition zone is the transparent zone surrounding the tested probiotics. The measurement was performed from the inner circle of the paper disk to the outer periphery of the disk [14].

2.7 Data Analysis

The data analysis of the inhibition zone of each tested group was done according to the statistical analysis test based on Shapiro-Wilk test, Levene's test, One Way Anova with the assistance of SPSS software (version 21, IBM Software, Armonk, NY, USA). Results were considered statistically significant at $p < 0.05$. Shapiro-Wilk Test normality test was used to observe whether the obtained data were normally distributed. The Levene's Test is a homogeneity variant test that was used to test the similarity of the variants of the samples. The test of dissimilarities was performed using One Way ANOVA to find whether there were significant dissimilarities among the diameters of the inhibition zone in all of the tested groups. The outcome was then tested further with the post hoc test (Tukey HSD).

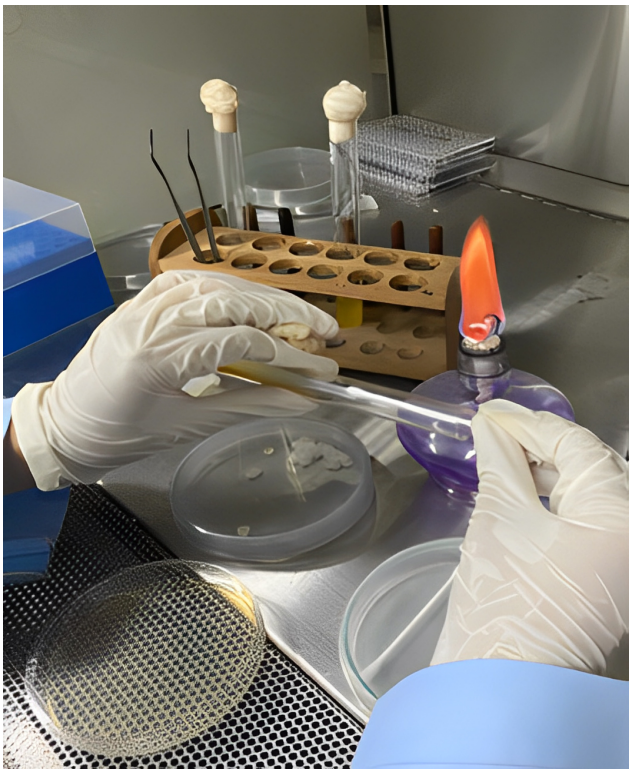

Fig. 2. *A. actinomycetemcomitans* ATCC 43718 serotype b were then planted on MHA using 3× cotton swabs at an angle of 60°.

Fig. 3. Paper disk was placed on MHA.

3. Results

Inhibition Zone Diameter of 3 Types of Probiotics Against *A. actinomycetemcomitans* stp. b: Bacteria growth after conducting the research, the inhibition data obtained

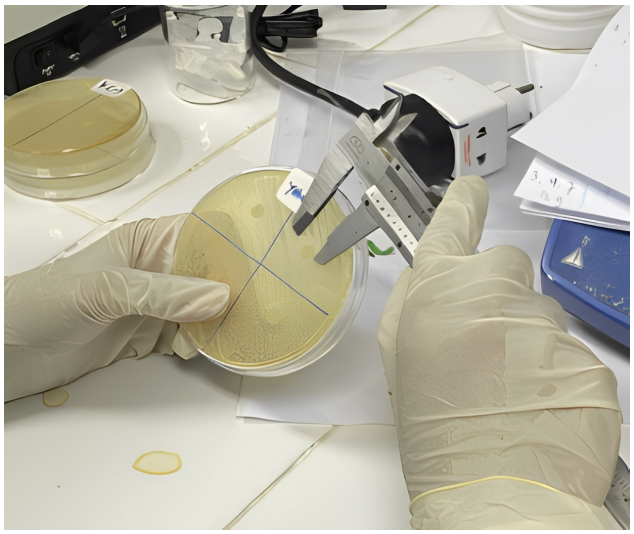

Fig. 4. The diameter measurement of the inhibition zone in each testing group was conducted with a Vernier caliper in millimeters.

among product A with *L. casei* strain Shirota, product B containing *L. bulgaricus* and *S. thermophilus*, as well as product C containing *L. reuteri* Prodentis, the researchers concluded that the probiotics worked against the growth of *A. actinomycetemcomitans* bacteria. The average value of inhibition zone diameter in product C was 19.60 ± 0.438 mm, in product B was 16.60 ± 0.591 mm, whereas in product A was 12.70 ± 0.694 mm. While the negative control did not show any inhibition zone at all. Table 1 and Fig. 5 shows the large inhibition zone formed on the MHA media created by the different test materials.

Table 1. The resulted inhibition zone diameters in the treatment group with the aforementioned probiotics from product A, product B, product C and control group.

Replication	Inhibition Zone (Diameter, mm)			
	Product C	Product B	Product A	Control (-)
1	19.60	16.80	13.20	0.00
2	19.80	17.30	13.15	0.00
3	19.60	16.70	13.30	0.00
4	18.93	15.88	11.95	0.00
5	19.38	15.90	12.90	0.00
6	20.25	17.05	11.70	0.00
Average	19.60 ± 0.438	16.60 ± 0.591	12.70 ± 0.694	0.00

The prerequisite to the ANOVA test was conducted with Shapiro-Wilk test to determine the normality of data distribution, which yeilded the significance (Sig.) of >0.05, meaning that the data was normal in its distribution. The results of the normality diameter zone of each treated group in accordance to Shapiro-Wilk test can be seen in Table 2.

The homogeneity test was performed with Levene's test. In turn the significance of (Sig.) 0.917, 0.292 and 0.078 were obtained for product C, B and A, meaning that the variant data were homogenous. The results of inhibiting zone diameter homogeneity of each treated group was in accordance with Levene's test with the significance of (Sig.) 0.260.

Table 2. The results of the normality diameter zone in each treated group in accordance to Shapiro-Wilk test.

Probiotics		Shapiro-Wilk test		
		Statistic	df	Sig.
Inhibition Zone	Produc C	0.974	6	0.917
	Product B	0.885	6	0.292
	Product A	0.814	6	0.078

ANOVA test was used to find out the difference between mean inhibition zone of three treatment groups. The result of the test revealed a significant difference (Sig.) 0.000, suggested that the mean inhibition zone of three treatment groups was significantly different.

In the post hoc test (Tukey HSD), the mean value of the group treatment which had been given product C was 2.98833 higher than that of in product B and 6.89333 higher than that the treatment given product A. The results of post hoc test (Tukey HSD) can be seen in Table 3.

4. Discussion

This study was intended to determine the effects and the differences in the probiotic inhibition, namely *L. casei* strain Shirota in product A (i.e., Yakult), *L. bulgaricus* and *S. thermophilus* in product B (i.e., Cimory Yogurt Drink), and *L. reuteri* Prodentis in product C (i.e., BioGaia Prodentis) in reducing the growth of bacteria named *A. actinomycetemcomitans*. In this study the statistical results obtained by ANOVA test were further supported the finding of the influence of probiotic in product A, product B, and product C compared to the control group. In addition, there was a notable difference in the inhibition zone diameter among the three probiotics. The anti-bacterial effect possessed by the aforementioned probiotics was capable of, indeed, inhibiting the growth of *A. actinomycetemcomitans*. This was influenced by the mechanism of the action of probiotic bacteria. Probiotic bacteria are lactic acid bacteria with strong inhibitor effect against gram-negative bacteria, and they also work as an antimicrobial agent to impede the activity of pathogenic bacteria. Additionally, the decrease of the intracellular pH of the organic acids contained within the probiotic bacteria can cause cell death of pathogenic bacteria. Probiotic bacteria produce antimicrobial peptides, including bacteriocins [25,26]. Bacteriocins are formed by gram-positive bacteria, which kills pathogenic bacteria by destroying target cells and inhibiting cell wall synthesis [27,28]. The diameters of the inhibition zone on the MHA media indicate whether the *A. actinomycetemcomitans* was resistant towards the probiotics. In this study, the inhibition

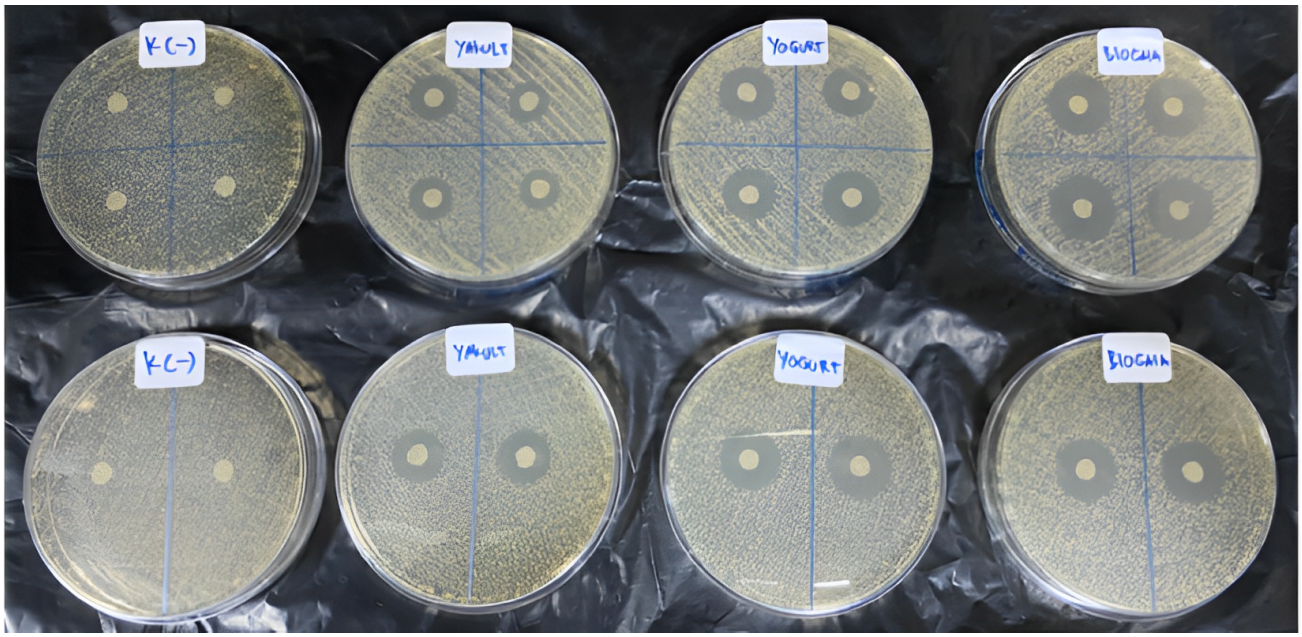

Fig. 5. The inhibition zone diameter dissimilarities in each tested group.

Table 3. The results of the post hoc test (Tukey HSD) inhibition zone diameter.

					95% Confidence interval	
Probiotic		Mean Difference	Std. Error	Sig.	Lower Bound	Upper Bound
Product C	Product B	2.98833	0.33748	0.000	2.1117	3.8649
	Product A	6.89333	0.33748	0.000	6.0167	7.7699

zone diameter was formed in the treatment groups which means that the *A. actinomycetemcomitans* bacteria were not resistant to probiotic bacteria. All three probiotics in product A, product B, and product C can slow down the growth of the bacteria. However, there was a significant difference in the inhibition effect among the three probiotics. The results of this analysis were different from the hypothesis due to the size of the inhibition zone formed not only by the strains of probiotic bacteria, but also by the concentration of antimicrobial compounds or substances, types of microbes, the number of microbes, pH, temperature and contact time. In this study there were differences in the types and numbers of microbes in the three probiotics. Therefore, the results of statistical analysis show an important difference in the aforementioned zone of inhibition [14]. Of the three treatment groups given probiotics, probiotic in product C had the largest inhibition zone diameter compared to product B and product A. This was because product C contains *L. reuteri* Prodentis bacteria. According to Jaffar *et al.* [29] *L. reuteri* as potential candidates, are effective in the treatment of oral diseases by repressing the development of periodontal pathogens. They serve as a good alternative due to the benefits that these organisms have to counteract pathogenesis by periodontal pathogens. As a matter of fact their ability to bring about an immunomodulatory response by an increase in cytokine production, an antiviral response against vesicular stomatitis by way of an interaction with macrophages, and also the induction of nitric oxide synthesis might give a strong effect against pathogens which tend to be virulent toward immune cells, as reported for *A. actinomycetemcomitans* previously. These bacteria include the *Lactobacillus* heterofermentative type, which later causes the glucose fermentation process, which in turn not only produces lactic acid, but also manufactures other components in equal amounts such as acetic acid, carbon dioxide (CO_2), and ethanol. This is an antimicrobial component that can inhibit the growth of pathogenic bacteria. Besides that, *L. reuteri* Prodentis creates two bacteriocins (reuterin and reutericyclin) as secretion [30,31]. It has antibacterial properties capable of inhibiting the development of various pathogenic bacteria as well as possessing a strong ability to compete with pathogenic bacteria. It has an anti-inflammatory properties able to inhibit the secretion of pro-inflammatory cytokines [22,23]. In an experiment done by Vivekananda *et al.* [23] indicated that *L. reuteri* Prodentis was indeed able to impede the growth of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* [32,33]. In an experiment done by Ikram *et al.* [34] have compared clinical efficacy of the local probiotic *L. reuteri* applied with toothbrush around gingival margin for 5 minutes twice a day with systemic amoxicillin 500 mg, in addition to SRP show improvement for all clinical periodontal parameters. In a

recent clinico-microbiological study conducted by Butera *et al*. [35] compared two new formulation of probiotics (in the form of toothpaste and chewing gum) against the chlorhexidine based toothpaste. On the basis of clinical and microbiologial findings, they conclude that probiotics tested in the study signify a valid support to scaling and root planing (SRP) with a benefit on several clinical parameters and on specific pathogenic bacteria causing periodontal disease. Another study conducted by Butera *et al*. [36] in 2022, they evaluated two new formulations of paraprobiotics (Biorepair Peribioma toothpaste and mouthwash) as an adjuct therapy to scaling and root planing. The used products caused a significant reduction in most of the clinical indices evaluated, and reduction in pathogenic bacteria constituting "Rex complex" which is significantly related to periodontal disease.

The probiotics in product B and product A contain homofermentative lactic acid bacteria. They in turn perform the glucose fermentation process which only produces lactic acid as the main component. However, the group treated with probiotic in product B had a larger diameter of the inhibition zone than the group treated byproduct A because the product B contained two lactic acid bacteria, namely *L. bulgaricus* and *S. thermophilus*. *L. bulgaricus* bacteria that produces amino acids and peptides as a source of nitrogen to form bacterial cells, so that more bacterial cells are produced. While *S. thermophilus* bacteria, apart from producing lactic acid, also produce formic acid. Albeit it is solely produced in small amount, it has a strong antimicrobial effect. Lactic acid and a little formic acid that are formed capable of inhibiting the growth of pathogenic bacteria because the presence of non-dissociating acid molecules that are able to penetrate cell walls and disrupt the metabolic process of pathogenic bacteria, furthermore a decrease in pH below the optimum pH for the growth of pathogenic bacteria [37]. The group treated with probiotic in product A had the smallest inhibition zone diameter compared to the group treated with probiotic in product C and product B. Although product B and product A both contain homofermentative types of lactic acid bacteria, product A only contains one lactic acid bacteria, that was *L. casei* strain Shirota. Thereof, the inhibition force of probiotic in product A was the smallest among the other two group treatments. Nevertheless, probiotic in product A could still inhibit pathogenic bacteria because *L. casei* strain Shirota contained was capable of lowering the local pH, inhibiting the growth of pathogenic bacteria because it produces antibacterial substances, that is bacteriocin [38,39].

5. Conclusions

The results of the current study revealed that all the purified bacteria from three probiotic treatment groups have growth inhibiting property on the periodontopathic bacteria *A. actinomycetemcomitans* ATCC 43718 serotype b starin. However, amongt the three treatment groups, *L. reuteri* Prodentis showed the highest inhibiting effect on the *A. actinomycetemcomitans* ATCC 43718 serotype b starin. Futher clinincal studies are required to support the findings of the current *in-vitro* study.

Availability of Data and Materials

The data supporting the finding is available with the corresponding author on the personal request to author.

Author Contributions

CPS, MS, and DFR designed the research study. MS, NU, SKS, and DFR performed the research. NU, SKS, IKEW, and SSA provided help and advice on data collection. SSA, CPS, IKEW, and PD analyzed the data. CPS, MS, NU, SKS, and SSA wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study received a Certificate of Ethical Eligibility from the Faculty of Dentistry, Universitas Airlangga Indonesia. Ethical clearance for the research was obtained from the Health Research Ethical Clearance Commission (No. 295/HRECC.FODM/VI/2020). All the experiments were conducted according to the Declaration of Helsinki.

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Conflict of Interest

The authors declare no conflict of interest.

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