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The Influence of Stationary and exponential Growth Phase of Probiotic Lactobacilli Towards *Aggregatibacter actinomycetemcomitans* Biofilm

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Abstract

Background: Bacterial biofilm of the oral cavity contributes to the dispersion of pathogenic organisms to other organs, particularly in immunocompromised patients. Lactobacilli own potent activity against the biofilm of the periodontal pathogen. The study aims to evaluate the inhibition activity of the probiotics lactobacilli's cells and supernatant during exponential and stationary phases against *Aggregatibacter actinomycetemcomitans*'s biofilm exponential phase.

Methods: Five Lactobacillus sp. and four *A. actinomycetemcomitans* strains were used during preliminary studies. Then, two chosen species of Lactobacillus sp. were used to determine inhibition activity towards *A. actinomycetemcomitans*'s biofilm using biofilm inhibition assay of a 96-well plate. Data of three replicates were presented as mean ± SD (standard deviation). The comparison was performed using Student t-test Software with P-value < 0.05 as the significant level.

Results: A significant difference in biofilm formation was observed in all four *A. actinomycetemcomitans* strains compared to the co-cultures biofilm assay with all probiotic lactobacilli for both cells and supernatant. All probiotic lactobacilli show biofilm inhibition activity. Interestingly, a significant difference ($p < 0.05$) was observed between the stationary and exponential phases in the inhibitory activity of *L. casei* NBRC 15883's cells. Whereas no significant difference was found for the biofilm inhibition activity of *L. casei* NBRC 15883's supernatant. Otherwise, there was no significant difference ($p > 0.05$) in inhibition activity between the exponential and stationary phase of *L. johnsonii* NBRC 13952 in both cells and supernatant.

Conclusion: This finding suggests a dynamic effect of the probiotic Lactobacillus sp. as part of counteraction strategies against the periodontal pathogen biofilm. The differential effect of stationary and exponential phases might indicate different mechanisms or compounds that require further study.

Introduction

Periodontal disease is an inflammatory state of the teeth affecting the periodontium. According to Nazir [1], periodontal diseases affect about 20-50% of the world's population in developing and developed countries. Global Burden of Disease Study [2] reported that half of the world's population (3.58 billion people) were affected by oral diseases. Periodontal disease is initiated by complex biofilm formation by periodontal pathogens. Later the condition worsens to chronic gingivitis and periodontitis.

Aggregatibacter actinomycetemcomitans is a non-motile Gram-negative pathogenic bacterium that inhabits the oral cavity. *A. actinomycetemcomitans* can be found in gingival crevices, dental plaque, and buccal mucosa, affecting up to 36% of the average population [3]. The periodontal pathogen has been used as a target organism due to the versatile virulence profiles owned by this group of pathogens.

Antibiotics have been widely used as treatment for several stages of periodontal disease. Examples of antibiotics commonly used are tetracyclines, doxycycline, metronidazole, amoxicillin, and ciprofloxacin [4]. However, established biofilms' complex and dynamic structure tolerates antibiotics due to restricted penetration towards the extracellular polymeric substances (EPS) biofilm layer [5]. Non-degradable residues of the administered antibiotics can lead to pollution. It has been reported that more than 80% of antibiotics are excreted as active metabolites in feces and urine [6]. Subsequent to this, antibiotic resistance genes were detected spreading in the exposed microorganism, thus elevating antibiotic concentration in the surrounding environment and resulting in the spread of multi-drug resistant bacteria. Non-surgical scaling and root planning may eliminate some periodontal species. However, it is often ineffective and does not considerably decrease the amount of *A. actinomycetemcomitans* [7].

There are several genera and many different species of lactic acid bacteria (LAB). They have the potential to be effective because they produce bacteriocin, enzymes that could prevent the production of biofilms, and bioactive peptides that could have a bactericidal effect against a wide range of microorganisms. [8].

Lactobacillus is one of the most important genera of LAB [9] that produce many compounds with an anti-microbial action, such as hydrogen peroxide, lactic acid, bacteriocin, and bacteriocin-like inhibitory substances [10]. They have been widely used in various applications, such as food processing and health care [11]. Probiotics can aid oral health by preventing the development of harmful microbiota or by modulating mucosal immunity in the oral cavity [12]. The pathways for probiotics activity in the oral cavity can be grouped into three major categories: Normalizing the oral microbiota, regulating the immune response, and metabolic impact [13]. This issue motivates this study to evaluate the dynamic potential of probiotic Lactobacilli against oral pathogens.

Methods

Strains and Culture Condition

One species of the periodontal pathogen was used in this study, which consists of four strains, *Aggregatibacter actinomycetemcomitans* Y4 (serotype b), SUNY 75 (serotype a), OMZ 534 (serotype e), and ATCC 29524. The periodontal pathogens were obtained from Kyushu Dental University, Japan. On the other hand, five probiotic strains of genus Lactobacillus were *L. casei* subspecies *rhamnosus* NBRC 3831, *L. fermentum* JCM 1137, *L. fermentum* NBRC 15885, *L. casei* NBRC 15883, and *L. johnsonii* NBRC 13952. All probiotics strains were obtained from the Kyushu Institute of Technology, Japan. All strains were stored at -80 ºC in appropriate culture media in a sterile 50% glycerol. Then, each strain of periodontal pathogen was stripped onto Brain Heart Infusion (BHI) agar supplemented with 1% yeast extract and incubated at 37 ºC for 48 h in anaerobic conditions. Then, the plate containing *A. actinomycetemcomitans* was sealed using parafilm and preserved in a chiller at 4 ºC. All probiotic strains were grown in De Man, Rogosa, and Sharpe (MRS) agar under anaerobic conditions at 37 ºC for 48 h using an anaerobic container. The plate containing Lactobacillus sp. was sealed using parafilm and preserved in a chiller at 4 ºC before being used in anti-microbial tests.

Growth Phase Determination

The exponential and stationary phases of Lactobacilli sp. were determined during the preliminary study. First, each strain of periodontal pathogen was cultured into Brain Heart Infusion (BHI) broth supplemented with 1% yeast extract and incubated at 37 ºC for 24 h in anaerobic conditions. Whereas, for the probiotic lactobacilli, all were grown in De Man, Rogosa, and Sharpe (MRS) broth under anaerobic conditions at 37 °C for 48 h by using an anaerobic container. Next, the concentrated stock was set to a new culture media to the initial OD 0.05 with the final volume of 30 mL. Next, the culture media were put into the anaerobic jar and incubated at 37°C. Absorbance was recorded every 2 hours.

Overlay Agar Method

Determination of the anti-microbial activity of each strain of probiotic against each pathogen was evaluated by using an overlay agar method. Two species of Lactobacillus sp. with the most significant and least significant were chosen for the biofilm inhibition assay experiment. First, a single colony of probiotics from the plate was isolated and cultured in De Man, Rogosa, and

Sharpe (MRS) broth with a volume of 10 mL. The culture was incubated for 24 h at 37 ºC in anaerobic conditions. This creates an optimal condition for the probiotic lactobacilli to grow and express their capacity to produce anti-*A. actinomycetemcomitans* components. Next, 3 µL of overnight culture (24 h at 37 ºC) of probiotic strains were spotted two times in distinct on the surface of plates containing 7 mL MRS agar (1.2%) as a first layer agar. Later, the spots were allowed to dry, and the plates were incubated for 24 h at 37 ºC under anaerobic conditions. The following day, a 200 µL sample of an overnight culture (24 h at 37 ºC) of *A. actinomycetemcomitans* was dropped into the 7 mL of warmed BHI soft agar (0.7%). Immediately, the mixture was poured over the plate surface containing the spots of probiotics to become the second layer of agar. Next, the plate was incubated for 24 h at 37 ºC under anaerobic conditions. For the negative control, *A. actinomycetemcomitans* was grown on the double-layer agar without the probiotics strain, and for the positive control, antibiotic tetracycline was used for comparison with probiotic Lactobacillus. Inhibition of *A. actinomycetemcomitans* growth was determined based on probiotic strains' inhibition zone formed around the spot. The inhibition of *A. actinomycetemcomitans* growth diameter was measured, and the petri dish was photographed.

Biofilm Inhibition Assay

Before this experiment, two species of probiotic lactobacilli cells (exponential and stationary phase) and all four *A. actinomycetemcomitans* strains (exponential phase) were cultured hours before the experiment following their preliminary growth phase data. Next, a biofilm inhibition assay followed Coffey and Anderson with some modifications [14]. The treatment was prepared by co-culturing 100 µL of each probiotic lactobacilli suspension into 100 µL of *A. actinomycetemcomitans* suspension in a 96-well plate. For the positive control, 100 µL of *A. actinomycetemcomitans* suspension was added to 100 µL of BHI broth, whereas for the negative control, the BHI broth without bacteria was used. The 96-well plates were incubated at 37 °C for 24 h, 48 h, and 72 h in anaerobic conditions. For the probiotic lactobacilli's supernatant preparation, the cultured cells were filtered first using a 0.45 µm syringe filter (Whatman Puradisc 25 Nylon) to separate the supernatant from the cells. Next, the same procedure for the biofilm inhibition assay was applied to the probiotic lactobacilli's supernatant. Ratio 1:1 of *A. actinomycetemcomitans* suspension to probiotic lactobacilli's supernatant was used for the co-culture assay.

Crystal Violet Staining and Biofilm Quantification

Biofilm inhibition was determined using 0.1% crystal violet staining. After incubation, the well was washed with distilled water three times to remove planktonic bacteria and leftover media from each well. The biofilm mass was determined by adding 200 µL of 0.1% crystal violet solution (w/v) to each well and dissolving for 30 min at room temperature [15]. Then, the plate was rinsed with distilled water to remove the crystal violet solution and allowed to air dry in an incubator at 37 °C for 15 min. Next, the stained biofilm was dissolved in 200 µl of 95% ethanol in each well for 30 min to solubilize the dye. Finally, the plate was read using a Tecan Infinite M200 Pro microplate reader at an absorbance of 492 nm. All the experiments were performed in triplicates, and the results were presented in the mean form.

Statistical Analysis

Data from at least three independent experiments were represented as mean ± SD (standard deviation). The comparison was performed using a student t-test using GraphPad Software, and P-value < 0.05 was considered significant.

Results

Growth phase determination of Lactobacilli sp. and *A. actinomycetemcomitans* strains

Figures 1 and 2 show the growth curve of each Lactobacillus sp. and *A. actinomycetemcomitans* strains used in this study. Table 1 shows the exponential and stationary phase timeframe for Lactobacillus species and *A. actinomycetemcomitans* strains. The average range of exponential phase for *A. actinomycetemcomitans* strains was between the 4th to 16th hours of culturing. For Lactobacillus species, the average range for the exponential phase was between the 12th to 24th hours, while stationary phase was between 20th to 32nd hour. The mid of range hours were selected for the cultured period during the biofilm inhibition assay experiment.

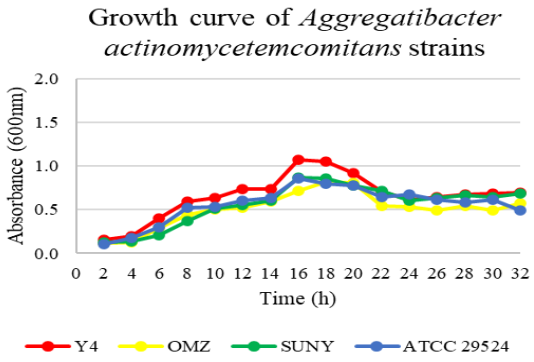

Figure 1: Growth curve of *A. actinomycetemcomitans* strains

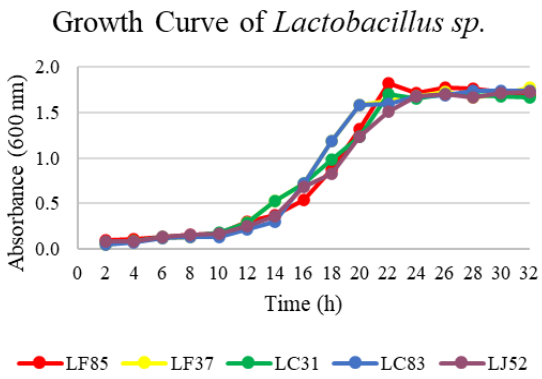

Figure 2: Growth curve of *Lactobacillus* sp.

The exponential phase of *A. actinomycetemcomitans* was cultured for the 12th hour, the exponential phase of Lactobacillus species was cultured for the 12th hour, and the stationary phase was for the 24th hour.

Inhibition zone of Lactobacillus sp. against *A. actinomycetemcomitans*

Based on Table 1, the inhibition effect of Lactobacillus sp. against *A. actinomycetemcomitans* strains was evaluated where antibiotic tetracycline was a positive control. Overall data showed the inhibition zone of antibiotic tetracycline against all four *A. actinomycetemcomitans* strains was higher than all four Lactobacillus sp. used in this study except *L. casei* 15883 against *A. actinomycetemcomitans* SUNY 75 strain. The antibacterial effects were classified into four categories based on the diameter of the clear zone formed, namely, weak (<0.4 cm), medium (0.4-0.8 cm), strong (0.8-1.2 cm), and very strong (>1.2 cm) [16]. Based on Table 2 data, the positive control, tetracycline, had a very strong antibacterial effect against all four strains of *A. actinomycetemcomitans*. All species of probiotic lactobacilli showed both very strong and robust antibacterial effects. *L. casei* NBRC 15883 showed a powerful antibacterial effect for all *A. actinomycetemcomitans* strains. This is followed by *L. fermentum* NBRC 15885, a very strong anti-bacterial against three out of four of *A. actinomycetemcomitans* strains. *L. fermentum* JCM 1137 and *L. johnsonii* NBRC 13952 showed a strong antibacterial effect against all four *A. actinomycetemcomitans* strains. Based on this data, for the next experiment, a very strong antibacterial effect of *L. casei* NBRC 15883 was put against *L. johnsonii* NBRC 13952, which has a strong antibacterial effect for comparison.

Biofilm inhibition activity of probiotics lactobacilli against *A. actinomycetemcomitans*

Based on biofilm inhibition activity data, co-culture of all four *A. actinomycetemcomitans* strains with lactobacilli cells and supernatant showed a significant difference ($p < 0.05$) against positive control *A. actinomycetemcomitans* throughout 72 h of incubation. Lactobacilli cells and supernatant inhibition activity was also compared to see if there was any difference between them. There was a significant difference ($p < 0.05$) between lactobacilli cells and supernatant against Y4 and SUNY 75 strains for both growth phases, where supernatant has higher inhibition than cells. Besides that, against OMZ 534 strain, stationary phase lactobacilli showed a significant difference ($p < 0.05$) between cells and supernatant. Nevertheless, exponential phase lactobacilli showed a significant difference ($p < 0.05$) for both cells and supernatant when against ATCC 29524 strain.

On the contrary, some showed no significant difference ($p > 0.05$) between cells and supernatant. Inhibition activity of exponential *L. johnsonii* NBRC 13952 showed no significant difference ($p > 0.05$) between cells and supernatant against OMZ 534 strain (serotype e). In addition to that is the stationary phase of both lactobacilli, which showed no significant difference ($p > 0.05$) between cells and supernatant when against the ATCC 29524 strain. Overall, most showed a significant difference ($p < 0.05$) between cells and supernatant of lactobacilli against the *A. actinomycetemcomitans* strain.

Influence of pH on biofilm inhibition activity

The pH was observed during biofilm formation. The pH value for positive control ranged between pH 7.33 to 8.18. In contrast, the co-culture treatments with lactobacilli6 cells showed higher acidic pH values ranging between pH 4.41 to 6.20 compared with lactobacilli supernatant; pH 5.26 to 6.19 across all four *A. actinomycetemcomitans* strains. Positive control showed an essential condition while co-culture treatments for both lactobacilli cells and supernatant in acidic conditions. The presence of lactobacilli might be the reason for the high acidity in the co-culture treatment of 96-well plates.

Differential effect of stationary and exponential phase toward biofilm inhibition activity

The P-value comparing both growth phases was measured to evaluate the significant difference between the exponential and stationary phases of the probiotic lactobacilli towards *A. actinomycetemcomitans* biofilm inhibition activity. Based on Table 2, both cells and supernatant of all probiotic lactobacilli showed no significant difference ($p>0.05$) during both exponential and stationary phases in their biofilm inhibition activity against all *A. actinomycetemcomitans* strains. However, a different finding was observed for *L. casei*

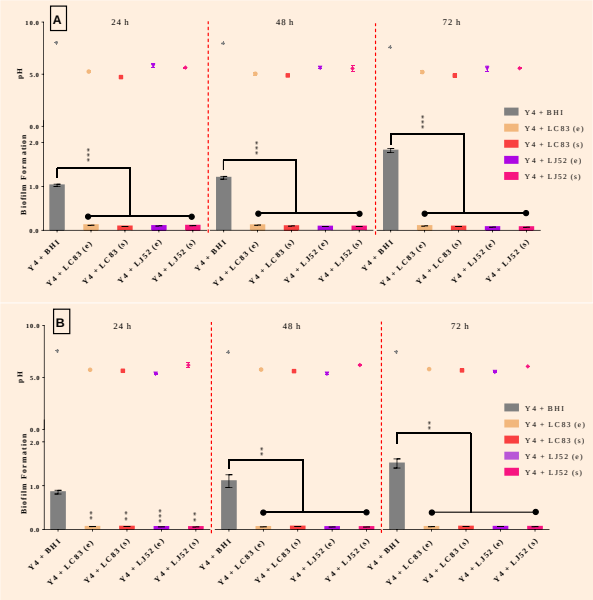

Figure 3: Biofilm inhibition activity of *A. actinomycetemcomitans*, Y4 strain (serotype b) against lactobacilli (A) cells and (B) supernatant for 24, 48, and 72 h. Control and probiotic species were labeled as follows; Y4: *A. actinomycetemcomitans* Y4 strain, LC83: *L. casei* NBRC 15883, and LJ52: *L. johnsonii* NBRC 13952. e = exponential phase, s = stationary phase. Bars represent the mean, error bars represent standard deviation and significance was measured using paired T-test (* = P< 0.05, ** = P< 0.01, *** = P<0.001).

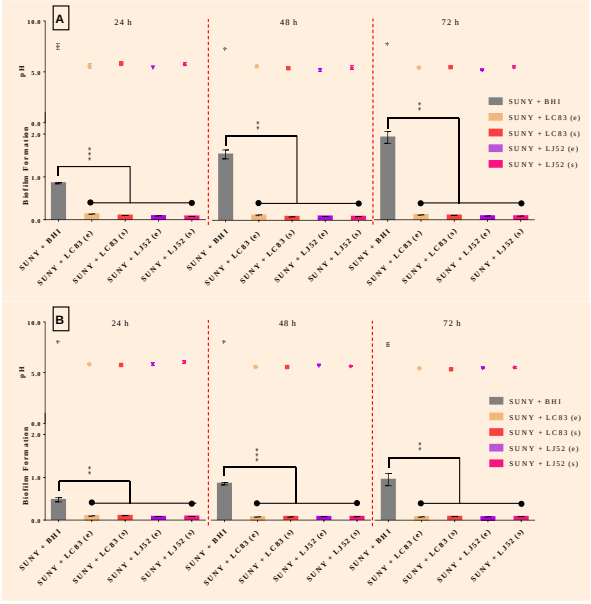

Figure 5: Biofilm inhibition activity of *A. actinomycetemcomitans*, SUNY 75 strain (serotype a) against lactobacilli (A) cells and (B) supernatant for 24, 48, and 72 h. Control and probiotic species were labeled as follows; SUNY: *A. actinomycetemcomitans* SUNY 75 strain, LC83: *L. casei* NBRC 15883, and LJ52: *L. johnsonii* NBRC 13952. e = exponential phase, s = stationary phase. Bars represent the mean, error bars represent standard deviation and significance was measured using paired T-test (* = P< 0.05, ** = P< 0.01, *** = P<0.001).

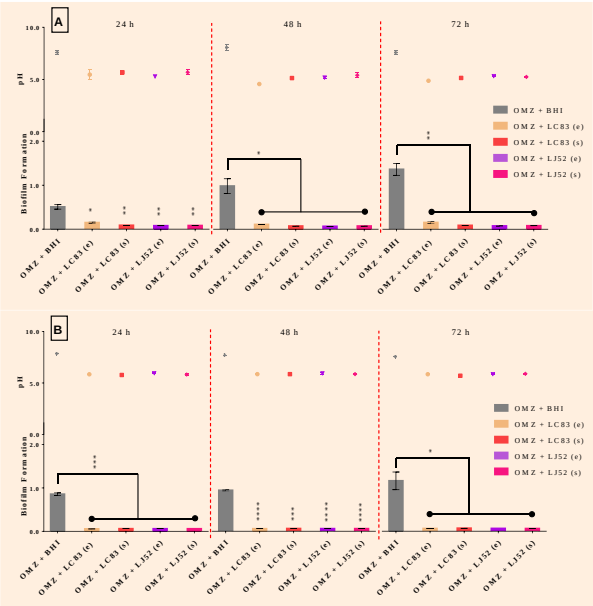

Figure 4: Biofilm inhibition activity of *A. actinomycetemcomitans*, OMZ 534 strain (serotype e) against probiotic Lactobacilli (A) cells and (B) supernatant for 24, 48, and 72 h. Control and probiotic species were labeled as follows; OMZ: *A. actinomycetemcomitans* OMZ 534 strain, LC83: *L. casei* NBRC 15883, and LJ52: *L. johnsonii* NBRC 13952. e = exponential phase, s = stationary phase. Bars represent the mean, error bars represent standard deviation and significance was measured using paired T-test (* = P< 0.05, ** = P< 0.01, *** = P<0.001).

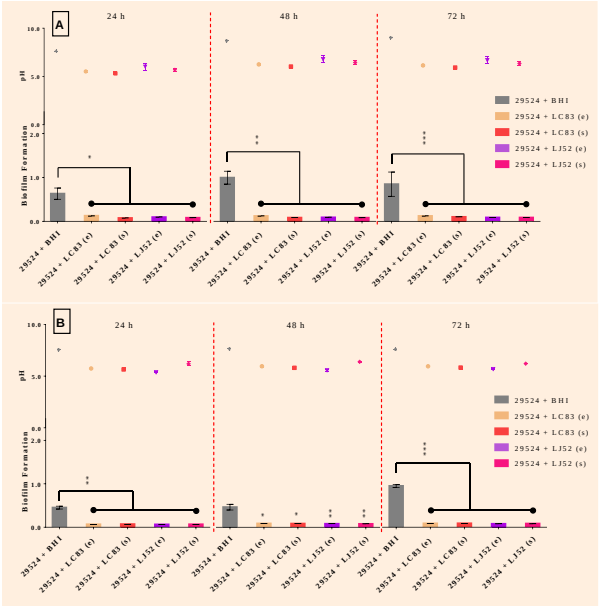

Figure 6: Biofilm inhibition activity of *A. actinomycetemcomitans*, ATCC 29524 strain against probiotic Lactobacilli (A) cells and (B) supernatant for 24, 48, and 72 h. Control and probiotic species were labeled as follows; 29524: *A. actinomycetemcomitans* ATCC 29524 strain, LC83: *L. casei* NBRC 15883, and LJ52: *L. johnsonii* NBRC 13952. e = exponential phase, s = stationary phase. Bars represent the mean, error bars represent standard deviation and significance was measured using paired T-test (* = P< 0.05, ** = P< 0.01, *** = P<0.001).

actinomycetemcomitans	Antibiotic disc (Tetracycline)	*L. casei* NBRC 15883	*L. fermentum* JCM 1137	*L. fermentum* NBRC 15885	*L. johnsonii* NBRC 13952
Y4	2.0	1.43 ± 0.05	0.97 ± 0.50	1.35 ± 0.13	0.83 ± 0.42
OMZ 534	1.4	1.30 ± 0.14	1.00 ± 0.58	1.20 ± 0.69	0.95 ± 0.06
SUNY 75	1.4	1.65 ± 0.19	1.08 ± 0.05	1.15 ± 0.21	1.00 ± 0.58
ATCC 29524	2.8	1.28 ± 0.15	0.88 ± 0.05	1.20 ± 0.08	0.95 ± 0.10

Table 1: The mean for the zone of inhibition (cm) ± standard deviation of different species of A. actinomycetemcomitans against probiotic Lactobacilli species. Tetracycline was used as a positive control

Oral pathogen	Period	Biofilm Formation (OD)							
		Lactobacilli cells				*Lactobacilli* supernatant			
		LC83 (e)	LC83 (s)	LJ52 (e)	LJ52 (s)	LC83 (e)	LC83 (s)	LJ52 (e)	LJ52 (s)
Y4	24 h	0.118*	0.088*	0.100	0.105	0.061	0.066	0.059	0.058
	48 h	0.116	0.093	0.084	0.086	0.060	0.069	0.058	0.058
	72 h	0.099	0.088	0.074	0.074	0.061	0.068	0.063	0.063
OMZ 534	24 h	0.149*	0.088*	0.078	0.082	0.055	0.060	0.059	0.059
	48 h	0.107**	0.072**	0.065	0.072	0.058	0.062	0.060	0.059
	72 h	0.153*	0.088*	0.075	0.079	0.062	0.070	0.064	0.061
SUNY 75	24 h	0.131*	0.101*	0.089	0.080	0.096	0.101	0.078	0.087
	48 h	0.115*	0.083*	0.092	0.086	0.073	0.079	0.078	0.080
	72 h	0.115	0.103	0.088	0.087	0.077	0.083	0.075	0.080
ATCC 29524	24 h	0.128*	0.075*	0.096	0.081	0.068	0.072	0.065	0.067
	48 h	0.122*	0.084*	0.090	0.082	0.088	0.086	0.082	0.079
	72 h	0.124*	0.102*	0.089	0.087	0.088	0.089	0.078	0.084

Table 2: Comparison of the biofilm formation during exponential and stationary phases of lactobacilli cells and supernatant against A. actinomycetemcomitans. The standard deviation and significance were measured between exponential and stationary phases using paired T-test (* = P< 0.05, ** = P< 0.01, *** = P<0.001).

NBRC 15883 cells which showed a significant difference ($p<0.05$) during exponential and stationary phases. Biofilm inhibition activity of *L. casei* NBRC 15883 cells is higher during the stationary phase for all four *A. actinomycetemcomitans* strains.

The presence of *L. casei* NBRC 15883 cells has a very strong antibacterial effect for all *A. actinomycetemcomitans* strains during the inhibition zone study (Table 1). A higher pH value in cells might contribute to the different inhibitory activity between the exponential and stationary phases of *L. casei* NBRC 15883.

Biofilm inhibition activity for both lactobacilli supernatant showed no significant difference ($p>0.05$) during the exponential and stationary phase against all four *A. actinomycetemcomitans* strains. The absence of lactobacilli cells might cause a depletion of byproduct production, especially in *L. casei* NBRC 15883, making both growth phases no different.

Discussion

Before the experiment started, preliminary growth curve was determined for all probiotics lactobacilli and *A. actinomycetemcomitans* strains. The study was focused on the exponential and stationary phases. The growth curve information helps decide the exponential and stationary phase for the probiotic lactobacilli and the exponential phase for *A. actinomycetemcomitans* strains. The exponential and stationery were chosen because cells in the exponential phase are preferentially used in industrial applications and research [17]. Furthermore, the growth rate increases during the exponential phase, cell doubling occurs at a relatively constant rate, and a uniform metabolic activity is produced. During the stationary phase, the growth rate begins to decline, and growth ceases due to nutrient exhaustion and the accumulation of toxic byproducts. Cells are still metabolically active and create secondary metabolites even while the growth rate is zero. During the stationary phase, the production of particular metabolites is increased due to metabolite deregulation [18]. This phenomenon may occur when the cells are not growing anymore but are metabolically active to secrete the secondary metabolites. Because of that, our interest is to evaluate those conditions' influence on biofilm inhibition activity.

Another preliminary study was conducted to choose two species out of five lactobacilli cells. The inhibitory effect of Lactobacillus sp. shows a promising inhibition area (strong and very strong antibacterial effect) against *A. actinomycetemcomitans*, although the area is less than positive control antibiotic tetracycline. Among five species of lactobacilli, *L. casei* NBRC 15883 showed a very strong antibacterial effect for all *A. actinomycetemcomitans* strains. *L. fermentum* JCM 1137 and *L. johnsonii* NBRC 13952 showed a strong antibacterial effect against all four *A. actinomycetemcomitans* strains. A study done by Husain *et al.* [19] showed the diameters of inhibition zones of B. subtilis strain under the exponential phase (7 hours incubation) and stationary phase (21 hours incubation) had a strong effect against S. aureus and are immensely affect strong against E. coli. At the same time, the effect of tetracycline is categorized as very strong against both E. coli and S. aureus. Prabhurajeshwar and Chandrakanth [20] showed that all

the isolated Lactobacillus strains produced the average inhibition (1.5-2.5 cm) pathogens' growth, whereas T2 (*L. fermentum*), T4, and T16 were the most effective against the growth of the test pathogens (2.8-3.2 cm). So for this study, *L. casei* NBRC 15883 and *L. casei* NBRC 15883 were chosen to determine the range of effectiveness against *A. actinomycetemcomitans*.

Antibacterial activity is part of the critical options for effective and novel probiotics. Figures 3,4,5, and 6 show that all data showed a significantly different ($p < 0.05$) between control *A. actinomycetemcomitans* strains and co-culture treatments with probiotics lactobacilli. One valuable finding in this study was that *A. actinomycetemcomitans*'s biofilm was inhibited in the presence of lactobacilli which confirmed their high inhibition activity. Moreover, data also confirmed inhibition activity occurred both during the exponential and stationary phases regardless of cells or supernatant. This result could be due to several mechanisms, including producing toxic compounds such as lactic acid, hydrogen peroxide, and bacteriocins [21] enhanced in probiotic lactobacilli. Some lactobacillus produces biosurfactants that can effectively prevent biofilm formation by adsorbing solid surfaces, impairing bacterial adherence [22]. Theoretically, compound production related to inhibitory substances peaked during the exponential and stationary phase of lactobacilli. Rezvani *et al*. [23] reported that lactic acid production was observed at both exponential and stationary growth phases for five Lactobacillus strains studied. The pH data support the statement since all treatments well during the biofilm inhibition assay were in an acidic medium-ranged between pH 4.33 to 6.38 for cells and 4.14 to 6.41 for supernatant, which correlated with the production of the acidic compound. A study by Prabhurajeshwar and Chandrakanth [20] shows pH values of Lactobacillus isolates in the range of 4.0 to 6.5, which aligns with the current study range. The highest acidity and lowest pH were observed after 72 h incubation at 37 °C for Lactobacillus sp. from all the collected samples. Todorov *et al*. [21] revealed that organic production increased with the incubation time, and the media's pH decreased with the increase of acid production. Schultze *et al*. [24] studied the impact of pH on the biofilm formation of the oral pathogen. In the biofilms representing periodontitis, at 24 and 48 h, the total counts of bacteria (log10 CFU), metabolic activity, and biofilm mass were lowest at pH 5 and 5.5. At the same time, the highest was detected at pH 7-8. This proved that during the acidic condition, periodontal metabolic activity was diminished.

For the comparison between probiotics lactobacilli's exponential and stationary phase, overall, for cells, there is a significant difference ($p < 0.05$) between the exponential and stationary phase, especially for *L. casei* NBRC 15883, which is substantial in all strains of *A. actinomycetemcomitans*. There is no significant difference ($p > 0.05$) for supernatant between exponential and stationary. A study by Rezvani *et al*. [23] showed that lactic acid production of *L. fermentum* was observed at both exponential and stationary growth phases, where maximum lactic acid concentration was obtained at the end of the stationary phase. A study done by Broeckx *et al*. [25] showed that Lactobacillus rhamnosus grown to the mid-log phase had a declining pH of 5.27 ± 0.02 to 3.99 ± 0.05 (17 h) and 3.65 ± 0.01 (24 h) during the stationary phase, which corresponds to the increase in lactic acid owing to the bacterial metabolism during growth. High lactic acid concentration might be the reason for the high acidity in the treatment 96-well plate.

For the comparison between cells and supernatant of probiotics lactobacilli, overall, there was a significant difference ($p < 0.05$) between cells and supernatant. The data showed that lactobacilli supernatant has a higher inhibition activity compared with cells. This finding proved that cells are not the factor inhibiting *A. actinomycetemcomitans*'s biofilm, but the compound or metabolites produced by lactobacilli. The early production of the biochemical compound by lactobacilli was high enough to inhibit *A. actinomycetemcomitans*'s biofilm, which was proven by the results where biofilm was inhibited during the first 24 h maintained throughout the 72 h incubation period. The focus now was biochemical compounds or metabolites responsible for inhibiting *A. actinomycetemcomitans*'s biofilm. Prabhurajeshwar and Chandrakanth's [20] study found no inhibitory activities of the supernatant of all three isolated Lactobacillus strains after being treated with trypsin and pronase. This finding might suggest that the inhibition effect was caused by the protein-like substance, for instance, bacteriocin produced by the Lactobacillus strains.

To summarize, our finding emphasizes the unique property of probiotic cells that plausible to be clinically effective therapeutics and directly interact with periodontal pathogen biofilms. However, more investigation and focused investigations must be carried out to clarify the mechanisms underlying this action. Comparing how probiotic bacteria and the periodontal pathogen interact with host cells may help develop effective methods to manage infections in vivo.

Competing Interest

The authors have declare that there is no conflict of interest.

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Author Contributions

Norzawani Jaffar and Kesaven Bhubalan designed the experiment and wrote the manuscript. Syakir Syahiran collected and analyzed the data and wrote the manuscript.

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