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Characterization and potential oral probiotic properties of *Lactobacillus plantarum* FT 12 and *Lactobacillus brevis* FT 6 isolated from Malaysian fermented food

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A R T I C L E I N F O

Keywords:
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lactic acid bacteria
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A B S T R A C T

Objective: This study aims to characterise the lactic acid bacteria (LAB) isolated from local Malaysian fermented foods with oral probiotics properties**.**
Design: The LAB strains isolated from Malaysian fermented foods, *Lactobacillus brevis* FT 6 and *Lactobacillus plantarum* FT 12, were assessed for their antimicrobial properties against *Porphyromonas gingivalis* ATCC 33277 via disc diffusion assay. Anti-biofilm properties were determined by treating the overnight *P. gingivalis* ATCC 33277 biofilm with different concentrations of LAB cell-free supernatant (LAB CFS). Quantification of biofilm was carried out by measuring the optical density of stained biofilm. The ability of *L. brevis* FT 6 and *L. plantarum* FT 12 to tolerate salivary amylase was also investigated. Acid production with different sugars was carried out by pH measurement and screening for potential antimicrobial organic acid by disc diffusion assay of neutralised probiotics CFS samples. In this study, *L. rhamnosus* ATCC 7469, a commercial strain was used to compare the efficacy of the isolated strain with the commercial strain.
Results: Lactobacillus brevis FT 6 and *L. plantarum* FT 12 possess antimicrobial activity against *P. gingivalis* with inhibition diameters of more than 10 mm, and the results were comparable with *L. rhamnosus* ATCC 7469. The MIC and MBC assay results for all tested strains were recorded to be 25 μl/μl concentration. All LAB CFS reduced biofilm formation proportionally to the CFS concentration and tolerated salivary amylase with more than 50% viability. Overnight cultures of all lactic acid bacteria strains showed a pH reduction and neutralised CFS of all lactic acid bacteria strains did not show any inhibition towards *P. gingivalis.*
Conclusions: These results indicate that the isolated probiotics have the potential as probiotics to be used as a supportive oral health treatment, especially against a periodontal pathogen, *P. gingivalis.*

1. Introduction

Probiotics are a beneficial group of microorganisms that will benefit the host when consumed in an adequate amount (Martín & Langella, 2019). Probiotics have been shown to be able to treat or prevent various types of conditions such as allergies, digestive problems, pathogen infections, and in some cases chronic liver diseases and diabetes (Abatenh, 2018). In the last decade, research has proven that probiotics are able to inhibit the attachment of related pathogens to the host tissue, thereby modulating immune responses in cases of dental infections and the production of an antimicrobial compound that could combat pathogenic oral bacteria. Probiotics reportedly have anti-biofilm properties and can disrupt the complex bacteria-to-bacteria communication (Pujia et al., 2017).

Fundamental research on probiotics has led to the development of a few strains that are now being tested in clinical trials to assist in the treatment of various dental issues (Pretzl et al., 2019; Yuki et al., 2019). The application of probiotics as an adjuvant therapy to non-surgical treatment such as scaling and root planing (SRP) for periodontal patients has been studied, and a few of the studies recorded positive outcomes such as reduction of periodontal pathogens and clinical improvements (Alanzi et al., 2018; Laleman et al., 2020).

Abbreviation: LAB, Lactic acid bacteria; CFS, Cell-free supernatant; MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration.

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Periodontitis or periodontal disease is a serious dental problem which affects around 50% of adults and adolescents in developing countries (Nazir, 2017). Periodontal disease is a chronic dental problem caused by the pathogenic invasion, which leads to the deterioration of the tissues surrounding the teeth and subsequently the loss of tooth attachment. This condition begins with the inflammation of the gingival tissue and progresses to its recession (Michaud, et al., 2017). Important periodontal pathogens are *P. gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Tannerella forsythia* (Alwaeli, 2018).

Among the aforementioned pathogens, *P. gingivalis* is the keystone pathogen that shifts the balance of oral microbiota and eventually disrupts the regulation of the immune response pathway. The shift in oral microbiota results in dysbiosis after the invasion of *P. gingivalis* (Curtis et al., 2020). There are a few *P. gingivalis* virulence factors that are functional for colonisation and recolonisation inside the oral cavity, the evasion of the immune responses and resistance towards antimicrobial treatment, including the fimbriae, gingipains, lipopolysaccharides (LPS) layer (Jia et al., 2019; Jun et al., 2017).

To protect against the recolonisation of *P. gingivalis* after mechanical treatments, a few established antibiotics and antibacterial mouthwash are administered to inhibit the pathogen. The most common antibiotics include metronidazole, amoxicillin–metronidazole combination, and azithromycin, whilst tetracycline, clindamycin, and minocycline are less common. Other than antibiotic drugs, antibacterial mouthwash chlorhexidine 0.2% is given to periodontal patients as adjunct treatment (Pretzl et al., 2019). There are a few drawbacks of the current antimicrobial treatments. They stain the tooth surface and reduce palatal sensitivity by chlorhexidine and antibiotics (Teoh et al., 2019; Zhao et al., 2020). Moreover, the biofilm formed by *P. gingivalis* with other periodontal pathogens reduces the efficiency of antibiotics treatment (Gerits et al., 2017).

Probiotics were introduced as a novel way to combat the biofilm formation of various pathogens (Coenye et al., 2020). Probiotics are a promising alternative to improve oral health, especially in gingival and periodontal disease management (Belibasakis et al., 2019). Numerous studies have found that periodontal pathogens can be inhibited by probiotics live culture or cell-free supernatant (CFS) from various commercial strains (Abdelhamid et al., 2018; Elgamily et al., 2018; Jeong et al., 2018). However, very few studies have characterised lactic acid bacteria isolated from fermented food as potential probiotics for oral health improvement.

In this study, *Lactobacillus brevis* FT6 and *Lactobacillus plantarum* FT12 isolated from fermented tapioca were investigated for their potential role as probiotics in oral health. The lactic acid bacteria candidates were screened for their antimicrobial susceptibility, and anti-biofilm formation properties and characterised for their probiotic potential. The isolated lactic acid bacteria were compared with the commercial strain *Lactobacillus rhamnosus* ATCC 7469.

2. Materials and methods

2.1. Bacterial strains

The two lactic acid bacteria (LAB) strains used in this study, namely *Lactobacillus brevis* FT 6 and *Lactobacillus plantarum* FT 12, were a private collection of lactic acid bacteria isolated from fermented foods in Malaysia (Abdul Rahman et al., 2020). *Lactobacillus rhamnosus* ATCC 7469 (ATCC, Manassas, VA, USA), a commercial probiotic, was included in the study as the positive control. All lactic acid bacteria strains were stored at -80 °C in 20% glycerol stock. The strains were revived by sub-culturing three times in Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, HA, UK) and incubated aerobically at 37 °C for 18 to 24 hours before being used for the experiment.

The pathogenic bacteria, *Porphyromonas gingivalis* ATCC 32377 (ATCC, Manassas, VA, USA) were streaked on Wilkins-Chalgren Anaerobic Agar (Oxoid, Basingstoke, HA, UK). The bacterial cultures were incubated anaerobically in an anaerobic chamber at 37 °C for 48 to 72 hours. The optimisation of the incubation period was also carried out by measuring the optical density, and observation of plate and microscopic morphology. All cultures were sub-cultured twice before use and were validated periodically by Gram staining procedures.

2.2. Preparation of cell-free supernatant

The cell-free supernatants were prepared using procedures adapted from a previous study (Abdelhamid et al., 2018; Tan et al., 2018). A single colony from an overnight culture of *L. brevis* FT 6, *L. plantarum* FT 12 and *L. rhamnosus* ATCC 7469 were grown into MRS broth media (Oxoid, Basingstoke, HA, UK). The cultures were incubated at 37 °C for 18 to 24 hours. The cells were then removed by centrifugation at 4000 g for 15 minutes. The supernatants were filter-sterilised with a 0.2 μm cellulose acetate filter (Thermo Scientific, MA, USA) to obtain the lactic acid bacteria cell-free supernatants (LAB CFS).

2.3. Antimicrobial activity screening

The antimicrobial activity was evaluated using the Kirby-Bauer disc diffusion assay procedure, which is based on the EUCAST protocol with modifications (Hindler et al., 2020). *Porphyromonas gingivalis* culture was grown overnight in Wilkins Chalgren broth media and diluted to McFarland 1.0 (Thermo, MA, USA). Next, 100 μL of the bacterial suspension was spread on Wilkins-Chalgren agar. The LAB CFS were then impregnated onto 6 mm Whatman filter paper and placed onto the agar. The plates were incubated in an anaerobic chamber for 48 hours based on the optimised growth observation at 37 °C. Commercial chlorhexidine 0.2% mouthwash (Rexidin, Indoco Remedies, Mumbai, India) was included as the positive control and sterile MRS broth was used as the negative control. All procedures were carried out in a Ruskinn Bugbox anaerobic chamber (5.5% hydrogen, 10% carbon dioxide, remainder nitrogen).

2.4. Antimicrobial susceptibility assay

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of LAB CFS were determined by broth microdilution assay based on CLSI methods (Weinstein & Patel, 2018). *Porphyromonas gingivalis* was grown overnight in broth media and diluted to McFarland 0.5. Then, 100 μL of bacterial suspension was transferred into a 96-well plate. The LAB CFS samples were diluted in sterile MRS broth that ranged from 0.03% to 10%, then were added into the well. Positive control chlorhexidine 0.2% and negative control sterile MRS were included in the test.

MIC was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Thermo Scientific, MA, USA). MBC values were determined by streaking the content of each well onto Wilkins-Chalgren Agar that had been divided into sections according to the concentration.

2.5. Anti-biofilm formation assay

The potential of isolated LAB CFS to inhibit biofilm formation was assessed by a procedure modified from previous studies (Brown et al., 2019; O'Toole, 2011). The pathogen was incubated overnight in a Wilkins-Chalgren broth medium and the culture was diluted into a fresh medium with a culture-to-medium ratio of 1:100. Then, 100 μL of the diluted bacterial solution was dispensed into each well and incubated for another 24 hours. The content of each well was discarded and rinsed with Phosphate Buffered Saline (PBS). 100 μL sterile Wilkins-Chalgren broth media were added to the well. 100 μL of LAB CFS samples at the MIC and MBC concentrations were added to the respective wells. The plates were incubated for 24 hours under anaerobic condition and the content of each well were discarded after the incubation period.

Staining and quantification of biofilm were done by rinsing each well with PBS followed by adding 125 μL of 0.1% crystal violet solution. After 15 to 30 minutes, the plates were rinsed twice to remove the unbound dye and were allowed to dry for an hour. 125 μL of 30% acetic acid was added to the dried 96-well plates to dissolve the stain followed by quantification using a microtiter plate at 550 nm wavelength.

2.6. Preliminary screening of potential antimicrobial organic acid

The antimicrobial organic acid screening was done by supplementation of 0.1 M NaOH solution into the LAB CFS until the pH of the LAB CFS reached pH 6.5 to pH 7. The neutralised CFS was used as the test sample following the procedures of disc diffusion assay. The controls for disc diffusion assay were used in this method.

2.7. Alpha-amylase viability screening

Alpha-amylase viability screening was carried out to assess the ability of the lactic acid bacteria strains to withstand oral amylase exposure. The assessment was carried out based on a previous study with slight modifications (Jeong et al., 2018). 1000 U/L of human salivary amylase (Sigma, St. Louis, MO, USA) was added into the MRS broth, which was then adjusted to pH 6.8. 0.5 ml of overnight LAB CSF were added into the modified MRS broth, incubated, and sampled at 0 and 4 hours. The cultures were diluted with PBS and plated onto MRS agar. The viability of the LAB cultures was assessed based on colony count at 4 hours when compared to 0 hours.

2.8. Autoaggregation and coaggregation assay

Autoaggregation assay for all LAB strains was carried out based on a reference method with some modifications (Kos et al., 2003; Li et al., 2020). All LAB were cultured for 18 hours at 37 °C in MRS broth. After the incubation period, the culture solutions were centrifuged at 4000 g for 15 minutes. The pellets were washed twice using PBS and resuspended into 2 ml sterile MRS broth to achieve 10^8 CFU/ml. The cell suspensions were vortexed to ensure uniform mixing and then incubated at room temperature for 5 hours. At every hour, 100 μl of the top layer of cell suspension was transferred into 3.9 ml of PBS. The absorbances of the suspension were measured at 600 nm and the autoaggregation percentage of each strain was calculated as 1 - (A_t / A_0) x 100, where A_t represents absorbance at each time t = 1, 2, 3, 4, or 5 hours and A_0 represent the absorbance at t = 0.

For the coaggregation assay, the cell suspensions were prepared similarly to the autoaggregation assay. Then, 2 ml of each cell suspension (lactic acid bacteria and pathogen) were transferred into a new tube and mixed by vortexing. Control tubes were prepared in the same manner, containing 4 ml of a single species cell suspension. Cell suspension samples were taken in the same way as autoaggregation assay and the absorbances were measured at 600 nm. The percentage of coaggregation was calculated using a reference formula (Li et al., 2020):

$$Coaggregation \quad \% = \frac{(Apro + Apat) - Amix}{(Apro + Apat)} \times 100$$

A_{pro}+ A_{pat} refers to the absorbance of probiotics and pathogens mixture at 0 hours and A_{mix} denotes the mixture of probiotics and pathogens at sampling time.

2.9. Acid production with different sugar supplementation

The determination of broth culture pH after the incubation period was carried out based on the reference method with slight modifications (Jeong et al., 2018). All probiotic strains were adjusted to 0.5% McFarland in sterile saline. The bacterial suspension (300 μL) was transferred into 10 ml sterile MRS broth supplemented with 4% of three types of sugars (sucrose, glucose, and fructose). The cultures were incubated overnight at 37 °C and the pH of the cultured broth was measured after the incubation period.

2.10. Statistical analysis

Data were reported in mean (±SD) and were analysed using SPSS Version 25 (IBM SPSS, Chicago, IL, USA). One-way ANOVA was used to compare the means of inhibition zone of different LAB CFS compared to control and the pH of the cultured broth for each type of sugar. The statistical significance was accepted with a p-value <0.05 with Tukey's HSD post hoc.

3. Results

3.1. Antimicrobial activity screening

The antimicrobial activity *of L. brevis* FT 6 and *L. plantarum* FT 12 have been assessed in this study. Both strains efficiently suppressed the growth of *P. gingivalis* with a similar effect. *Lactobacillus brevis* FT 6 had the largest inhibition zone (10.44 mm) followed by *L. plantarum* FT 12 CFS (10.22 mm). Their performances were comparable to the commercial *L. rhamnosus* ATCC 7469 CFS (10.26 mm) (Table 1). The highest inhibitory activity is shown by positive control, 0.2% Chlorhexidine (16.88 mm) which is used in the current clinical setting. The negative control (sterile MRS broth) showed no inhibition at all as expected.

3.2. Antimicrobial susceptibility testing of the probiotics supernatant

The MIC and MBC values of the lactic acid bacteria CFS samples were determined. *Lactobacillus brevis* FT 6 recorded a higher MIC and MBC value of 50 μl/μl (Table 2). The MIC and MBC values for *L. plantarum* FT12 and *L. rhamnosus* ATCC 7469 recorded the same value (MIC and MBC at 25 μl/μl). The MIC and MBC values of ampicillin and 0.2% Chlorhexidine were recorded at less than 3.125 μl/μl.(Fig. 1)

3.3. Anti-biofilm formation

The CFS *L. brevis* FT 6 (78.12% reduction at MIC concentration) strain recorded a higher percentage reduction of biofilm than *L. plantarum* FT 12 (76.12% reduction at MIC concentration) and *L. rhamnosus* ATCC 7469 (76.63% reduction at MIC concentration) (Fig. 2). The 0.2% chlorhexidine (76.62% at 0.125 MIC concentrations) recorded the highest overall biofilm reduction. The results were statistically significant at $p<0.05$ compared to the untreated biofilm with Tukey's HSD post hoc test.

3.4. Potential antimicrobial organic acid preliminary screening

The LAB CFS was then screened for compounds that could potentially be antimicrobial. The results showed that the CFS of *L. brevis* FT 6 and *L. plantarum* FT 12 did not inhibit *P. gingivalis* after being neutralised (Table 3). On the other hand, the CFS of *L. rhamnosus* ATCC 7469 showed slight inhibition after being neutralised. The controls showed consistent results as recorded in the preliminary screening.

Table 1
The inhibition diameter of the test samples against *P. gingivalis*.

Test Samples	Inhibition diameter, Mean±SD (mm)
L. brevis FT 6 CFS	10.44 ± 1.10 mm*
L. plantarum FT 12 CFS	10.22 ± 0.49 mm*
*L. rhamnosus*ATCC 7469 CFS	10.64 ± 0.44 mm
0.2% chlorhexidine	16.88 ± 2.16 mm
Sterile MRS broth	-

The size of Whatman paper is 6 mm. Experiments were conducted in triplicate and values are represented as mean ± SD. *Statistically significant with p-value<0.05 compared to positive control with Tukey's HSD post hoc analysis.

Table 2
The Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of LAB CFS against *P. gingivalis*.

Test Samples	Minimum Inhibitory Concentration, MIC (µl/µl)	Minimum Bactericidal Concentration, MBC (µl/µl)
L. brevis FT 6	50	50
L. plantarum FT 12	25	25
*L. rhamnosus*ATCC 7469*	25	25
0.2% Chlorhexidine*	<3.125	<3.125
Ampicillin	<3.125	<3.125
Sterile MRS broth	>100	>100

* Control

3.5. Alpha-amylase tolerance screening

The selected strains were assessed against alpha-amylase as a measure of resistance to oral enzymatic stress. Generally, all the tested strains were tolerant to alpha-amylase (Table 4) with variable resistance. *Lactobacillus plantarum* FT 12 recorded lower alpha-amylase tolerance with only 50% to 90% viability after being exposed to alpha-amylase. *Lactobacillus brevis* FT 6 and *L. rhamnosus* ATCC 7469 recorded the highest tolerance towards alpha-amylase exposure with more than 90% viability before and after the incubation period.

3.6. Autoaggregation and Coaggregation Assay

Different strains of lactic acid bacteria had shown different autoaggregation levels (Fig. 3). In the study, *L. plantarum* FT 12 recorded the highest autoaggregation percentage while *L. brevis* FT 6 recorded the lowest after 5 hours of incubation.

Moreover, *L. brevis* FT 6 showed the highest coaggregation activity with *P. gingivalis* (Fig. 3). *Lactobacillus rhamnosus* ATCC 7469 exhibited the lowest coaggregation activity with *P. gingivalis* at the different time points for 5 hours.

3.7. Production of acid with different sugars

Since lactobacilli are associated with the progression of dental caries, it is important to investigate the production of acid by selected strains when supplemented with different types of sugar. The pH values of the cultures dropped significantly after 24 hours of incubation (Table 5). The pH values for each lactic acid bacteria culture with different types of sugar were found to be in a similar range compared to the commercial strain. This showed that the isolated strains produced acidic compounds after 24 hours of incubation.

Other than that, supplementation of different types of sugar was shown to have no significant difference in the acid production of lactic acid bacteria, which proved that the intake of extra sugars would not affect the pH of the supernatant.

4. Discussion

Current antimicrobial treatments for periodontal disease are just sufficient to overcome colonisation, but they are not very effective at inhibiting the recolonisation of putative pathogens and they exhibit multiple side effects (Gerits et al., 2017; Jiao et al., 2019). The periodontal pathogen highlighted in this study, *P. gingivalis*, possesses multiple virulent factors and biofilm activity that contribute to its continuous infection on the gingival site (Bostanci & Belibasakis, 2012; Jia et al., 2019; Xu et al., 2020). Moreover, the rise in antibiotic resistance highlights the dire need for alternative treatment options.

Supportive treatments derived from various sources are investigated as a potential strategy to manage the pathogen progression in the disease. One highlighted strategy involves the development of probiotics as the potential adjunct therapy for periodontal disease management (Gerits et al., 2017).

Therefore, in this study, lactic acid bacteria isolated from local fermented foods are investigated for their applicability as oral probiotics against the keystone periodontal pathogen, *P. gingivalis. Porphyromonas gingivalis* is a highly putative pathogen that is involved in the progression of periodontal disease. This pathogen possesses multiple virulent factors including lipopolysaccharide (LPS) layer in its cell wall, fimbriae for adhesion, biofilm formation, gingipains for tissue degradation and multiple others that contribute to its detrimental effect and persistence in the oral environment (Jia et al., 2019; Xu et al., 2020).

The cell-free supernatant (CFS) of isolated probiotics, *L. brevis* FT 6 and *L. plantarum* FT 12, prepared in this study exhibited antimicrobial and anti-biofilm activity against *P. gingivalis* that are comparable with the commercial probiotic, *L. rhamnosus* ATCC 7469. These results agreed with another study that assessed the antimicrobial properties of *Lactobacillus curvatus* SMFM2016-NK isolated from fermented foods against periodontal pathogens such as *P. gingivalis* KCTC 5352 and *F. nucleatum* KCOM 1250, and *A. actinomycetemcomitans* KCOM1299 (Choi et al., 2021). This indicated that lactic acid bacteria isolated from fermented food have the potential to be antagonistic against periodontal pathogen. *P. gingivalis* had been reported to be susceptible in a few studies,

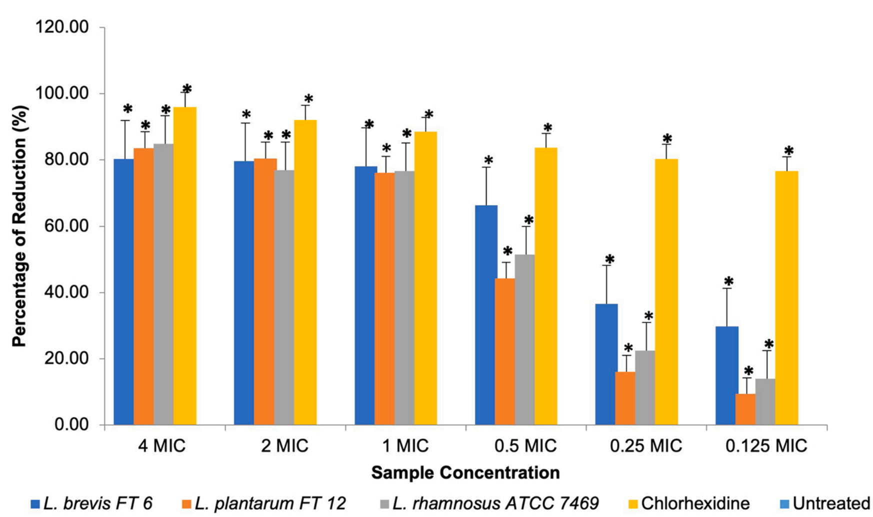

Fig. 1. Biofilm reduction percentage by different LAB CFS samples at different concentrations (significant percentage reduction with p <0.05 compared to untreated sample is denoted by *, standard deviations of each result are denoted in the error bar meanwhile standard deviation for all reduction percentage= 35.64).

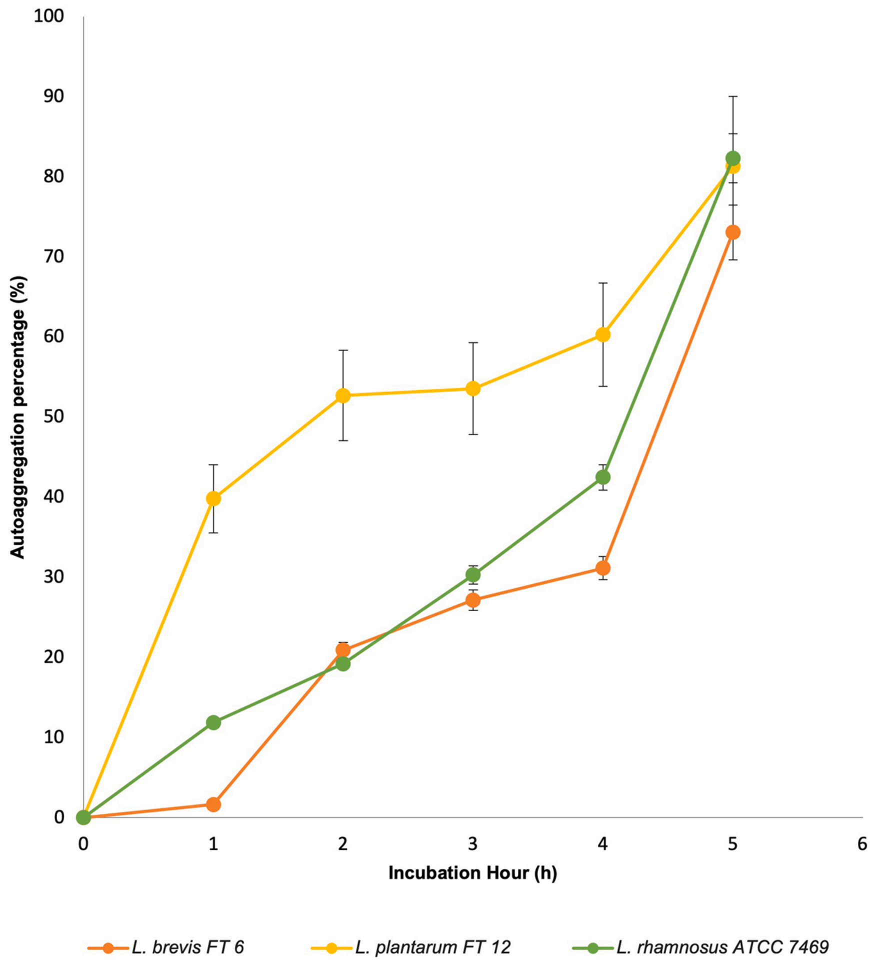

Fig. 2. The autoaggregation percentage of lactic acid bacteria strains during 5 hours of incubation. However, there is no statistical significant difference in the autoaggregation percentage between different strains.

Table 3
The alpha amylase tolerance of LAB CFS.

Probiotics Species	Alpha amylase tolerance, 0 hour	Alpha amylase tolerance, 4 hours
L. brevis FT 6	+++	+++
L. plantarum FT 12	+++	++
L. rhamnosus ATCC 7469	+++	+++

(+++ more than 90% viability, ++ 50% to 90% viability, + less than 50% viability, - no viable cell).

Table 4
Characterization of pH with different sugars ($p < 0.05$).

	Fructose	Sucrose	Glucose	Without Sugar
Before Incubation	6.22	6.19	6.24	6.26
After Incubation				
L. brevis FT 6	3.91	3.88	3.84	3.93
L. plantarum FT 12	4.05	3.85	3.91	3.98
*L. rhamnosus*ATCC 7469	4.11	4.14	4.05	4.11

particularly when being treated with live probiotics from commercial strains such as *S. dentisani, L. fermentum,* and *L. salivarius* (Chen et al., 2012; Esteban-Fernández et al., 2019). In addition, the commercial strain *L. rhamnosus* ATCC 7469 was reported to possess inhibitory activity against several periodontal pathogens such as F. *nucleatum, A. actinomycetemcomitans* and *P. gingivalis* (Moman et al., 2020). The mechanism of antagonistic activity against oral pathogens was postulated through competitive interaction as well as the secretion of antimicrobial substances such as hydrogen peroxide, organic acids, bacteriocin substances, and other bioactive compounds (Bustamante et al., 2020).

Once the strains have demonstrated some antimicrobial activity, the next course of action is to investigate their anti-biofilm activity. The disruption of biofilm produced by *P. gingivalis* is critical because periodontal disease is a biofilm-reliant disease that progressed with the establishment of biofilm on gingival tissue (Baek et al., 2018). The ability of LAB CFS to disrupt the biofilm formation activity at different concentrations is crucial to prevent the recolonisation of *P. gingivalis* as the protective biofilm layer in the pocket is destroyed by treatment (Bostanci & Belibasakis, 2012). In this study, the biofilm assay showed that the CFS of *L. brevis* FT 6, *L. plantarum* FT 12 and *L. rhamnosus* 7469 could disrupt the biofilm formation of *P. gingivalis* which would help to eliminate a major part of *P. gingivalis* pathogenesis. The result strongly agreed with another study that studied the anti-biofilm activity of *L. brevis* BBE-Y52 against multiple periodontal pathogens including *P. gingivalis* (Fang et al., 2018). In a detailed study, the researchers explored the effect of the CFS on *L. acidophilus* LA5, *L. rhamnosus* HN001, *L. reuteri* DSM 17938, *B. breve* 110, *B. pseudolongum* 119, and *B. bifidum* 162 on the biofilm-related gene expression of *P. gingivalis.* The study concluded that the CFS from the stated probiotics affected a few genes that are involved in biofilm formation (Ishikawa et al., 2020).

The production of potent antimicrobial materials that can disrupt the biofilm proved that the LAB CFS isolated from fermented foods is very

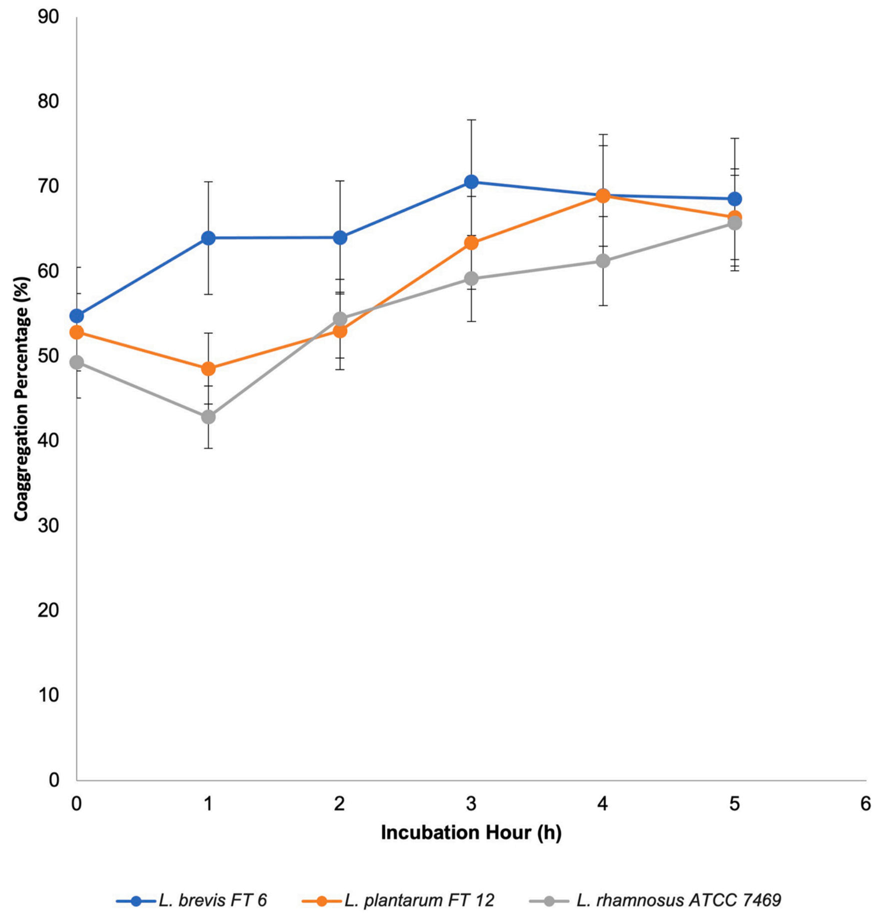

Fig. 3. The coaggregation percentage of lactic acid bacteria strains with *P. gingivalis* during 5 hours of incubation (There is no statistical significant difference in the coaggregation percentage of different strains).

Table 5
The inhibition activity of neutralized LAB CFS against *P. gingivalis*.

Test samples	Inhibition Activity
L. brevis FT 6	-
L. plantarum FT 12	-
*L. rhamnosus*ATCC 7469	+
0.2% Chlorhexidine	++
Sterile MRS broth	-

(-, no inhibition activity, +, <10 mm inhibition diameter, ++, 10 - 20 mm inhibition diameter, +++, >30 mm diameter)

competent at combatting the colonisation and biofilm formation of *P. gingivalis.* There is also a possibility that the metabolites produced by the isolated probiotics could affect cell wall synthesis, cell metabolism, and cell attachments.

One of the significant elements that might contribute to the antimicrobial activity of probiotics CFS is organic acid production. In this study, the preliminary screening for the activity of organic acids in the probiotic CFS was studied. The disc

diffusion assay showed that the neutralised CFS of *L. brevis* FT 6 and *L. plantarum* FT 12 did not show inhibition activity. On the other hand, the neutralised CFS of *L. rhamnosus* ATCC 7469 had shown slight inhibition towards *P. gingivalis* in the screening. This supports the assumption that organic acid might contribute to the antimicrobial activity of the tested LAB CFS. In support of this, a study reported that neutralised *P. acidilactici* HW01 CFS did not exhibit any antimicrobial activity against *Candida albicans* (Kim & Kang, 2019). The underlying element might relate to the direct antagonistic activity of organic acid or the supportive action of acidic conditions to activate bacteriocin-like compounds that are active at low pH (Shokri et al., 2018). The implication that organic acid might be the underlying component that exhibited bactericidal activity against *P. gingivalis* was supported by another study where low surrounding pH and moderate concentration of lactic acid in the environment affect *P. gingivalis* survival (Higuchi et al., 2019).

One of the postulated antimicrobial mechanisms of organic acid activity against pathogens is based on its ability to neutralise the cytoplasmic membrane of bacterial cells, rupturing it (Loh et al., 2017). Other than that, the presence of organic acid can also interfere with the quorum sensing of pathogens. Quorum sensing is a significant element in biofilm formation, especially in Gram-negative bacteria biofilm formation. Production of organic acids may also interfere with the integrity of rhamnolipid productions, a type of bacterial surfactant that plays a role in quorum sensing by *P. aeruginosa* (Kiymaci et al., 2018).

To counter the activity of *P. gingivalis* as a biofilm-forming pathogen, the autoaggregation and coaggregation activity of the studied LAB were observed. Good coaggregation activity of lactic acid bacteria with the pathogenic strain is important in preventing the pathogen from adhering to the gingival tissue and in displacing the pathogenic strain on the surface of healthy tissue. This characteristic is important to eliminate pathogens adhering on tissues and replace them with beneficial probiotic colonisation (Li et al., 2020).

In this study, *L. brevis* FT 6 and *L. plantarum* FT 12 were observed to have autoaggregation and coaggregation activity. In a previous study, *L. plantarum* isolated from Korean kimchi showed considerable autoaggregation activity which is in line with our observation (Khan & Kang, 2016). The autoaggregation activity is important in amplifying the probiotic's action (Li et al., 2020; Valeriano et al., 2014). The coaggregation activity result agreed with another study on the coaggregation activity of *L. rhamnosus* with *P. gingivalis,* it was reported that the probiotics could coaggregate with *P. gingivalis* after four hours (Mendi et al., 2016), which was also observed in our study.

The probiotic strains were also investigated for their ability to survive under harsh conditions. The probiotic isolates were assessed for their alpha-amylase tolerance, such that they can resist the lytic property of the lysozyme of mouth saliva. In this study, *L. brevis* FT6 and *L. plantarum* FT12 were recorded to be tolerant to alpha-amylase. This characteristic is important for the probiotics to persist in a viable state in various conditions so that they can be developed as an adjunct therapy (Lim et al., 2020). Similar findings were shown in another study, where probiotics isolated from kefir demonstrated excellent tolerance towards alpha-amylase exposure (Jeong et al., 2018).

The strains were also tested for their acid production when supplemented with various sugars. There was only a slight pH reduction after overnight incubation, indicating that the production of acid by the tested strains was not affected by the sugar supplementation. This metabolic activity is important because high acid production might cause dental caries (Pahumunto et al., 2020). A similar finding was previously reported by Jeong et al. (2018).

In conclusion, CFS isolated from fermented food, *L. brevis* FT 6 and L. plantarum FT 12, exhibited excellent antimicrobial and anti-biofilm activity against *P. gingivalis* and the results are comparable with CFS of commercial probiotics, *L. rhamnosus* ATCC 7469. The isolated lactic acid bacteria strains were also tolerant toward external stressors such as salivary amylase and showed excellent autoaggregation activity and moderate coaggregation activity with *P. gingivalis.* The acid production of LAB CFS was not affected by the sugar supplementation. The major contributor to the antimicrobial activity might be the organic acid produced by the probiotic amongst other antimicrobial elements.

CRediT authorship contribution statement

Nor Zaihana Abdul Rahman: Conceptualization; Nurul Szawani Mohd Zubri: Data curation; Nurul Szawani Mohd Zubri; Formal analysis; Nor Zaihana Abdul Rahman, Kalavathy Ramasamy: Funding acquisition; Nor Zaihana Abdul Rahman, Nurul Szawani Mohd Zubri: Investigation; Nor Zaihana Abdul Rahman, Nurul Szawani Mohd Zubri, Kalavathy Ramasamy: Methodology; Nor Zaihana Abdul Rahman: Project administration; Nor Zaihana Abdul Rahman: Resources; Nor Zaihana Abdul Rahman: Supervision; Nor Zaihana Abdul Rahman Kalavathy Ramasamy: Validation; Nurul Szawani Mohd Zubri, Nor Zaihana Abdul Rahman: Roles/Writing – original draft; Nor Zaihana Abdul Rahman Kalavathy Ramasamy: Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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