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In Vitro Antimicrobial Activity and Aggregation Abilities of Probiotic *Lactobacillus casei* and *Lactobacillus salivarius* Against Oral Pathogens

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ABSTRACT

The antagonistic effect of probiotics against oral pathogens merits exploration because these bacteria are beneficial to the host's health. The antimicrobial activity of two probiotic strains, *Lactobacillus casei* and *Lactobacillus salivarius*, as well as *L. casei* and *L. salivarius* combination (1:1), was investigated against *Streptococcus mutans*, *Streptococcus sobrinus*, *Candida albicans*, *Candida glabrata* and *Candida tropicalis* using agar-well diffusion, auto-aggregation and coaggregation assays. *L. salivarius* cell-free supernatant (CFS) alone exhibited greater inhibitory effect against *Streptococci* spp. compared to *L. casei* CFS alone and the combination. However, no inhibition was observed for *Candida* spp. *L. salivarius* alone exhibited significantly stronger auto-aggregation than *L. casei* alone ($p \leq 0.05$) and *L. casei* and *L. salivarius* combination. *L. salivarius* exhibited strong coaggregation ability with *Candida* spp., followed by *Streptococci* spp. while *L. casei* exhibited coaggregation only with *Streptococci* spp. However, *L. casei* and *L. salivarius* combination did not display any coaggregation with all strains. *L. salivarius* alone exhibited a stronger antagonistic effect on the tested organisms than *L. casei* alone or in combination. Based on the results, both probiotic strains showed good antimicrobial activities against oral pathogens and should be further studied for their human health benefits.

Keywords: *Agar diffusion; auto-aggregation; coaggregation; Lactobacillus spp.; oral pathogens*

INTRODUCTION

Lactobacilli are lactic acid bacteria group which produce various antimicrobial substances that exhibit antagonistic activity against pathogenic organisms. *Lactobacillus casei* and *Lactobacillus salivarius* are among probiotic bacteria found in the gastrointestinal tract and oral cavity, respectively that exert therapeutic properties. Probiotics are described as "beneficial microorganisms which when administered in sufficient quantities, improve the micro-ecological balance of the host and provide the host with a health benefit" (Hill *et al*., 2014). Several studies have indicated that

probiotic bacteria could be used in the treatment and prevention of oral diseases such as periodontal diseases and dental caries (Wu *et al.*, 2015; Jeong *et al.*, 2018).

Probiotics are more effective in interacting with the host to maintain homeostasis, which traditional therapies cannot achieve (Allaker & Stephen, 2017). They have great potential to inhibit pathogenic organisms through several mechanisms, such as pH alteration, antimicrobial compounds production, regulation of microbial pathogen growth through antagonism, compete for pathogen receptor binding sites, stimulate production of lactase by immune modulatory cells and suppress low-grade inflammation (Monteagudo-Mera *et al.*, 2019). In addition, probiotics acts as physical protective barrier by forming biofilm to protect against oral diseases. Probiotic bacterial adherence to oral tissues and enhance local immunity are another factor that promote the health of the host (Alok *et al.*, 2017).

Many bacterial strains used as probiotics have the ability to aggregate and coaggregate, which plays an important role in the formation of biofilms to protect the host from pathogen colonisation. Aggregation ability is correlated with cell adherence properties. Some probiotic strains can inhibit the pathogens adherence to intestinal mucosa either by direct coaggregation with pathogens or by forming a barrier via auto-aggregation (Choi *et al.*, 2018). Some probiotic lactobacilli species play important roles in microflora equilibrium and natural immunity in a variety of environments (Teanpaisan *et al.*, 2011), and increase the concentration of excreted antimicrobial substances in the process of coaggregating (Kaewnopparat *et al.*, 2013). The ability of bacterial strains to coaggregate is essential, as it can allow lactic acid bacteria strains to inhibit the growth of pathogenic strains in some ecological niches, such as the oral cavity. *Lactobacillus* probiotic bacteria present in yogurt have previously been shown to inhibit the growth and biofilm formation of *Streptococcus mutans* (Javid *et al.*, 2015; Wu *et al.*, 2019).

L. casei is a Gram–positive facultative heterofermentative bacteria, while *L. salivarius* is a Gram–positive obligately homofermentative bacteria. *Lactobacillus* species have been shown to have antimicrobial properties that can inhibit the growth of a variety of microbial pathogens (Jeong *et al.*, 2018). Two strains of *L. salivarius*, K35 and K43, was demonstrated to inhibit the growth and expression of *S. mutans* virulence genes and reduced this pathogen's biofilm formation (Wu *et al.*, 2015). *L. casei* ATCC 11578 influences the adherence of Streptococci to saliva-coated hydroxyapatite and release the already-bound Streptococci from hydroxyapatite (Stamatova & Meurman, 2009).

While these studies yielded promising results, research on the effects of *L. casei* and *L. salivarius*, as well as their combinations, on oral pathogens is still limited. Most studies were primarily focused on the impact of probiotics on enteropathogens. By modifying the biofilm composition of the oral cavity, probiotics may be used as an alternative in preventing and treating oral infectious diseases (Jiang *et al.*, 2016). Thus, this research was aimed to evaluate the antagonistic effects of *L. casei* and *L. salivarius* against several oral pathogenic strains (*S. mutans*, *S. sobrinus*, *C. albicans*, *C. glabrata* and *C. tropicalis*). All microorganisms used in this study are the most common pathogens associated several oral diseases, i.e., dental caries and candidiasis. Mutans streptococci (*S. mutans* and *S. sobrinus*) are considered to be major etiologic agents of dental caries, while Candida species are the most common causes of oral candidiasis (Pfaller *et al.*, 2010).

MATERIALS AND METHODS

An *in-vitro* experimental study was carried out at Medical Microbiology and Parasitology Laboratory, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia.

Test Microorganisms

Probiotic strains (*L. casei* ATCC 15883 and *L. salivarius* ATCC 11741), and oral pathogenic strains (*S. mutans* ATCC 25175, *S. sobrinus* ATCC 33478, *Candida albicans* HV27460, *C. glabrata* HV 27228 and *C. tropicalis* B27658) used in this study were commercially obtained from the American Type Cell Culture (ATCC, US).

Lactobacillus Cell-Free Supernatant

Preparation of *L. casei* and *L. salivarius* cell-free supernatant (CFS) was done as described by Coman *et al.* (2014). Each probiotic *Lactobacillus* strains was cultivated in De Man, Rogosa and Sharpe (MRS) broth (Oxoid, US) at 37°C for 24 h. Crude CFS of *Lactobacillus* strains were collected by centrifugation at 12,000× g for 20 min at 4°C and sterilised by filtration using 0.2 µm porous membranes. The final concentration of each *Lactobacillus* CFS was prepared to be 10^8 CFU/ml.

Antimicrobial and Antifungal Activities

In this study, antibacterial and antifungal activities of *L. casei* and *L. salivarius* and their combinations were evaluated using three different modified procedures: agar-well diffusion, auto-aggregation and coaggregation assays.

Agar-Well Diffusion

Agar-well diffusion test was done as described by Coman *et al.* (2014) with slight modifications of the tested probiotic where in this study, CFS of *Lactobacillus* strains was used instead of bacterial suspension. All pathogenic strains (*S. mutans*, *S. sobrinus*, *C. albicans*, *C. glabrata* and *C. tropicalis*) were lawn cultured over Brain-heart Infusion (BHI) agar (Oxoid, US). A 6 mm diameter wells were punched into agar plates and filled with 100 µl (10^8 CFU/ml) CFS of *L. casei*, *L. salivarius*, combination of *L. casei* + *L. salivarius* (1:1 ratio). Distilled water and 0.2% chlorhexidine gluconate were used as negative and positive controls, respectively. After incubation at 37°C for 48 h, the diameters of zone of inhibition (in mm) were measured using a digital calliper. This experiment was done in triplicates. The antimicrobial activity was recorded as growth-free inhibition zones measured from the edge of the wells.

Auto-Aggregation and Coaggregation Assays

Auto-aggregation refers to bacteria's self-binding and self-recognition capacity, which can be seen macroscopically as bacterial clumps form at the bottom of culture tubes. Coaggregation, on the other hand, is the tendency of various bacterial strains to associate (Trunk *et al.*, 2018). These abilities are critical for adherence to epithelial cells and the development of biofilms to protect the host from pathogens. In the present study, auto-aggregation and coaggregation assays were adapted from a previous study (Prabhurajeshwar & Chandrakanth, 2017).

Briefly, *L. casei* and *L. salivarius* were cultivated in MRS broth at 37°C for 24 h. After centrifugation at 6000× g for 20 min at 4°C, the pelleted cells were subsequently washed three times with sterile phosphate buffer solution (PBS) (pH 7.2). The cells were then resuspended in PBS to a final concentration of 10^8 CFU/ml. One hundred microlitre of *Lactobacillus* suspension and its combination (ratio of 1:1 v/v) were mixed by vortexing, followed by incubation at 37°C for 4 h without agitation. The absorbance, A_{600} was determined at 0 h (A_{0hr}) and 4 h (A_{4hr}). This experiment was done in triplicates. The percentage of auto-

aggregation was calculated using the following formula:

$$\text{Auto-aggregation} = 1 - [(A_{4hr} / A_{0hr})] \times 100\%$$

For the coaggregation assay, the suspension of *Lactobacillus* and oral pathogenic strains were prepared as described in the auto-aggregation assay. The suspension of *Lactobacillus strains* and their combination were mixed with oral pathogen suspensions (ratio of 1:1). The mixture was then incubated at 37°C for 4 h without agitation. This experiment was done in triplicates. The absorbance was determined at 0 h and 4 h at 600 nm. The coaggregation percentage was calculated using the following formula:

$$\text{Coaggregation} = [(A_{pathogen} + A_{lactobacillus})/2 - A_{mix} (A_{pathogen} + A_{lactobacillus})/2] \times 100\%$$

where $A_{pathogen}$ and $A_{lactobacillus}$ represent the absorbances measured from of each strain, while A_{mix} represents the absorbance measured from the mixture of the pathogen and *Lactobacillus* strains.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics 25 software (IBM Corp., NY). The data were assessed using ANOVA for agar-well diffusion test, Kruskal-Wallis test for auto-aggregation and Mann-Whitney test for coaggregation. The significance level was set at $p < 0.05$. The data were presented as mean ± standard deviation (SD).

RESULTS

Antimicrobial Activity of *Lactobacillus* CFS Using Agar-Well Diffusion

Both *Lactobacillus* CFS demonstrated antimicrobial activity against *S. mutans* and *S. salivarius*, notable by the presence of inhibition halos around the wells (Fig. 1). *L. salivarius* alone exhibited better inhibition towards *S. mutans* and *S. sobrinus* compared to *L. casei* alone (Figs. 1a and 1b). However, no inhibition was observed for all *Candida* spp. (Figs. 1c–1e). The mean diameters of the inhibition zone exhibited by the *Lactobacillus* CFS on the tested pathogenic strains are presented in Table 1. When compared statistically among *L. salivarius*, *L. casei* and their combination, there is no significant difference ($p > 0.05$) between inhibition zones observed among both *Streptococcus* spp.

Auto-Aggregation and Coaggregation Assays of *Lactobacillus* Spp.

As shown in Fig. 2, *L. salivarius* alone exhibited the highest auto-aggregation ability (80.41%±0.19), compared to *L. casei*

Table 1 Mean zone of inhibition probiotic *L. casei*, *L. salivarius* CFS using agar-well diffusion

Oral pathogenic strains	Positive control (0.2% Chlorhexidine gluconate)	Negative control (distilled water)	Mean diameter of inhibition (mm)		
			L. salivarius	*L. casei*	*L. casei* + *L. salivarius*
S. mutans	31.33±0.83 (+++)	–	24.50±0.75 (+++)*	12.50±1.53 (++)*	22.50±0.40 (+++)*
S. sobrinus	24.17±0.90 (+++)	–	17.67±0.72 (++)*	13.67±0.70 (++)*	15.33±1.13 (++)*
C. albicans	20.67±1.42 (+++)	–	–	–	–
C. glabrata	20.00±1.34 (+++)	–	–	–	–
C. tropicalis	20.83±1.24 (+++)	–	–	–	–

Note: *One-way ANOVA test ($p > 0.05$)
– no inhibition; + zone of inhibition less than 10 mm (low inhibition); ++ zone of inhibition 10–20 mm (intermediate inhibition); +++ zone of inhibition more than 20 mm (strong inhibition)

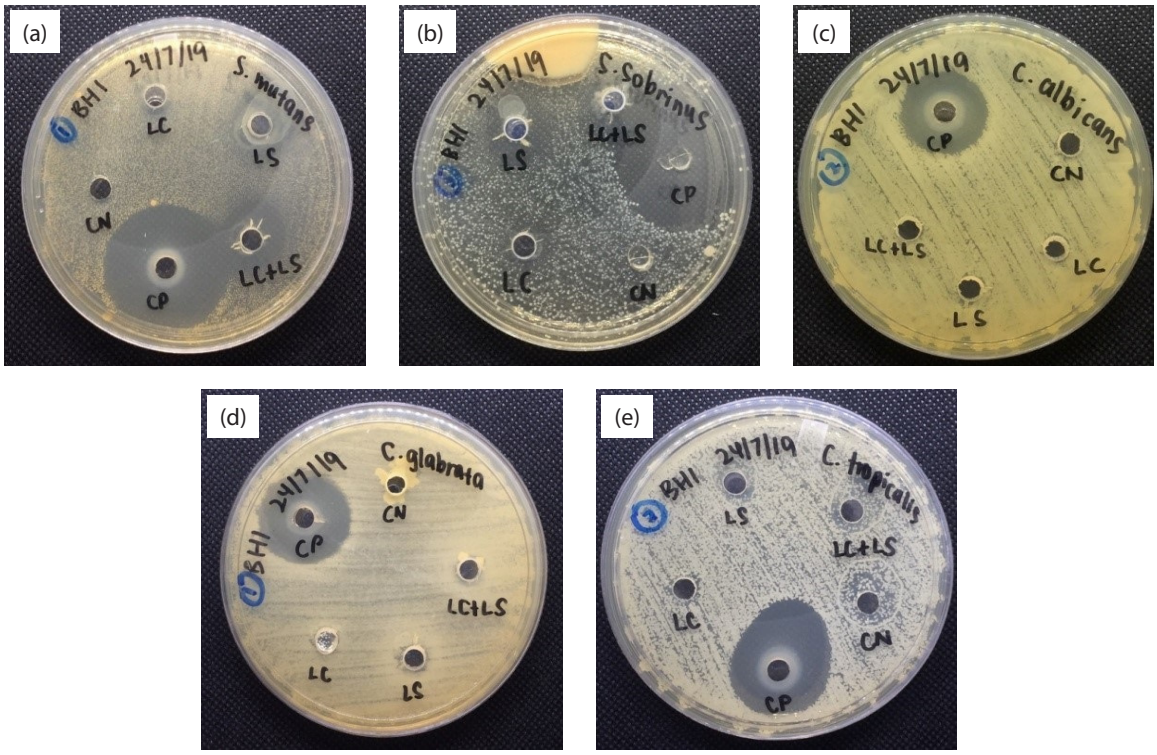

Fig. 1 Inhibitory effects of probiotics strains tested by agar-well diffusion method (a: *S. mutans*, b: *S. sobrinus*, c: *C. albicans*, d: *C. glabrata*, e: *C. tropicalis*, CP: 0.2% chlorhexidine gluconate, CN: distilled water, LC: *L. casei*, LS: *L. salivarius,* LC+LS: *L. casei* + *L. salivarius*).

alone (28.14%±0.89) after 4 h. However, combination of *L. salivarius* + *L. casei* exhibited lower auto-aggregation ability (68.37%±1.09) as compared to *L. salivarius* alone. Among the oral pathogenic strains, lower auto-aggregation ability was demonstrated by *S. mutans* (9.62%±1.22) and *S. sobrinus* (6.71%±0.68) as compared to the *Candida* spp. Among the *Candida* spp., *C. tropicalis* exhibited the highest auto-aggregation ability (43.15%±0.52). The percentage of auto-aggregation for all oral pathogenic strains ranged between 6%–43%, which is a 2-fold below the range of percentage of auto-aggregation for *Lactobacillus* strains and its combination (28%–80%). There was a statistically significant difference in auto-aggregation potential between *L. salivarius* and *L. casei* alone (Kruskal-Wallis test, $p = 0.027$). However, no significant different was observed among *L. salivarius* or *L. casei* alone and *L. salivarius* + *L. casei* combination ($p = 0.539$).

The coaggregation ability of *Lactobacillus* spp. with different oral pathogens is shown in Table 2. Among the tested strains, *L. salivarius* had similar coaggregation ability with both *Streptococci* spp. where the percentage of coaggregation against *S. mutans* was 26.55%±1.08 and against *S. sobrinus* was 21.00%±0.73. *L. salivarius* had a higher percentage of coaggregation ability against *Streptococci* spp. than *L. casei*. *L. salivarius* also exhibited slightly higher coaggregation ability against *Candida* spp. than against *Streptococci* spp. Among all the pathogenic strains, *L. salivarius* demonstrated the least coaggregation against *S. sobrinus* (21.00%±0.73) and the most coaggregation ability against *C. tropicalis* (33.48%±0.63). *L. casei* showed no coaggregation against all *Candida* spp. *L. casei* showed higher coaggregation ability against *S. mutans* (17.32%±0.35) than *S. sobrinus* (14.28%±0.75). However, the combination of both *Lactobacillus* spp. did not show any coaggregation with all tested oral pathogens.

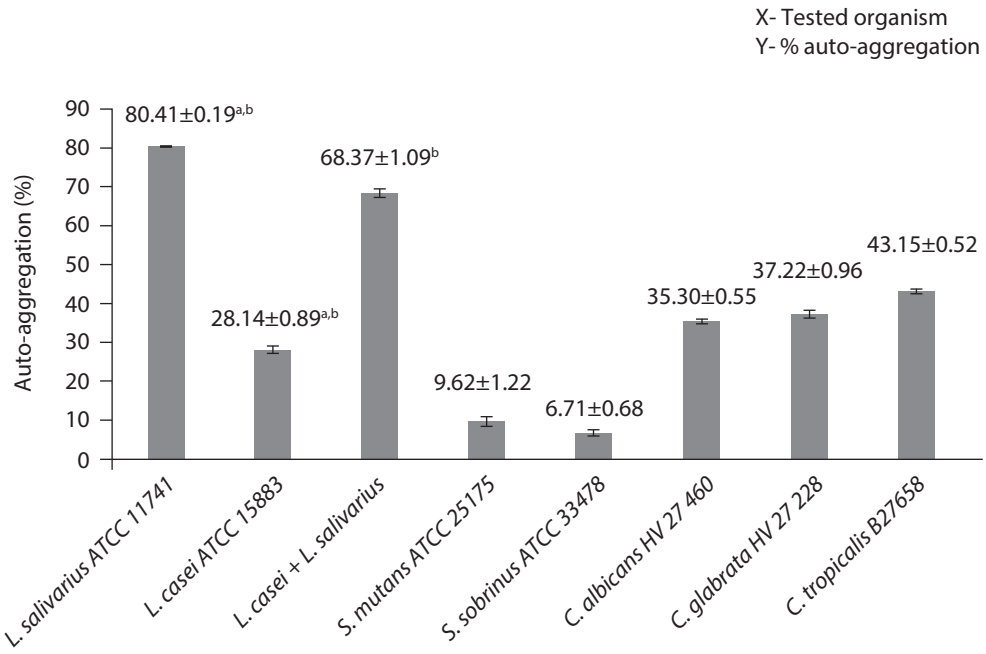

Fig. 2 Auto-aggregation of *L. casei*, *L. salivarius* and oral pathogenic strains. [a] There was a statistically significant difference in auto-aggregation potential when comparing between *L. salivarius* and *L. casei* alone ($p \leq 0.05$; Kruskal-Wallis test). [b] No significant difference was observed between *L. salivarius/L. casei* alone and their combination (*L. salivarius* + *L. casei*) ($p \geq 0.05$; Kruskal-Wallis test).

Table 2 Coaggregation of *L. casei* and *L. salivarius* against oral pathogenic strains

Oral pathogenic strains	Coaggregation (%)		
	L. salivarius	*L. casei*	*L. casei + L. salivarius*
S. mutans	26.55±1.08[a,b]	17.32±0.35[a]	–
S. sobrinus	21.00±0.73[a,b]	14.28±0.75[a]	–
C. albicans	28.13±1.02[b]	–	–
C. glabrata	30.63±0.62[b]	–	–
C. tropicalis	33.48±0.63[b]	–	–

Notes: – no coaggregation; [a] There was a significant difference in coaggregation ability of *Lactobacillus* spp. on *Streptococcus* spp. (Mann-Whitney test; $p \leq 0.05$); [b] No significant difference in the coaggregation ability of *L. salivarius* against oral pathogenic strains (Mann-Whitney test; $p > 0.05$).

Statistically, there was a significant difference ($p = 0.05$) between coaggregation effects of *L. salivarius* and *L. casei* with *Streptococci* spp. However, no significant difference was observed in coaggregation ability of *L. salivarius* with all tested pathogenic strains ($p = 0.499$).

DISCUSSION

To date, the emergence of antibiotic-resistant bacteria has been a major source of concern for global health. As a result, probiotics with beneficial effects may be a feasible option for addressing this problem. Probiotics have been used as an adjunct to scaling and root planing, to improve clinical gingival bleeding and probing depths, as well as to minimise oral malodor in patients with

chronic periodontitis and halitosis (Penala *et al.*, 2016), implying their possible use in the treatment or prevention of oral diseases such as periodontal diseases and dental caries (Naghmouchi *et al.*, 2020). Several previous studies reported that *Lactobacillus* spp. have an antimicrobial effect against various pathogens, but they primarily focused on enteropathogens (Prabhurajeshwar & Chandrakanth, 2017; Tebyanian *et al.*, 2017; Chen *et al.*, 2019). The study on the effects of *L. casei* and *L. salivarius*, as well as their combinations, on oral pathogens are scarce. Thus, the present study was aimed to determine the antagonistic effect of these two probiotic strains and their combinations against selected oral pathogenic strains.

In this study, the probiotic *L. casei* and *L. salivarius* strains possess varying degrees of antibacterial and antifungal activities towards the oral pathogenic strains. The results of inhibitory activity vary between different methods used, revealing different antimicrobial mechanisms. In agar-well diffusion method, both *Lactobacillus* strains inhibited the growth of *S. mutans* and *S. sobrinus*. The finding was in line with other studies which reported the inhibition of *S. mutans* growth by *L. salivarius* (Wu *et al.*, 2015; Krzyściak *et al.*, 2017; Lin *et al.*, 2017). However, in this study both CFS *Lactobacillus* strains and the combination did not exhibit any inhibition against *Candida* spp. In a previous study, Radi *et al.* (2015) demonstrated a low inhibition of *Lactobacillus* bacteria against *Candida* spp. However, in another study by Song & Lee (2017) demonstrated strong antifungal activity of *L. casei* (ATCC 334) against blastoconidia and hyphal form of *C. albicans*, and inhibited *Candida* biofilm on the denture base resin. These variations could be due to different components presence in the bacterial suspension and CFS and different strains/ isolates used in the study.

The CFS contains several metabolites and amino acids with high antimicrobial and antioxidant activities, as determined by the GC–MS (Shehata *et al.*, 2019). Previous studies reported that lactic acid and protein (bacteriocin) molecules in the CFS have antimicrobial properties (Hladíková *et al.*, 2012; Alvarez-Sieiro *et al.*, 2016). Lactic acid penetrates and disrupts the pathogen cell membrane due to its pH reduction and undissociated nature, resulting in the breakdown of the by-layers and transmembrane proton motive power force (Alakomi *et al.*, 2000). The acidification of the membrane by lactic acid could have also enhanced the antimicrobial activities of other biomolecules such as diacetyl, which may require a low pH environment to function. Gram-positive bacteria possess a thick cell wall made up of teichuronic or teichoic acid polymers. These highly anionic lipid components that are exposed on the bacterial membrane structures are ideal targets for the cationic antimicrobial peptides (Omardien *et al.*, 2016). Bacteriocins, on the other hand, are closely cationic active compounds that easily interact with anionic lipid components of the membrane, resulting in the creation of pores that facilitate cell lysis (Oscáriz & Pisabarro, 2001). This mechanism could explain why bacterial species with higher anionic lipid content are more susceptible to the antibacterial effect exhibited by these cationic active compounds.

The combination of both *Lactobacillus* CFS demonstrated antagonistic affect and greater inhibition against *S. mutans* than *L. casei* alone, but less inhibition compared to *L. salivarius* alone. Antagonism is thought to be one of the mechanisms for the action of probiotic bacteria. This antipathogenic activity involves competitive exclusion and the production of antimicrobial compounds. In addition, the antagonistic effect of probiotics against oral pathogens may also be attributed to different mechanisms of action, such as biosurfactant production, adhesion and coaggregation (Monteagudo-Mera *et al.*, 2019).

The auto-aggregation and coaggregation abilities of *Lactobacillus* spp. and the oral pathogenic strains were investigated in this study because adhesion ability is an

essential property of the probiotics to undergo transient colonisation. This helps to facilitate immunomodulatory effects and prevents the adherence of pathogens to epithelial receptors (Monteagudo-Mera *et al.*, 2019). In the present study, *Lactobacillus* probiotic strains exhibited twice a range of percentage of auto-aggregation compared to oral pathogenic strains. This result was consistent with a previous study (Prabhurajeshwar & Chandrakanth, 2017), which found that probiotic strains had a 2.5-fold auto-aggregation capacity as compared to oral pathogenic strains. Lactobacilli with high autoaggregation ability showed high hydrophobicity (Chen *et al.*, 2010; Nikolic *et al.*, 2010), and as a result better adherence to the cells. Auto-aggregation is one of the first steps in the formation of biofilm and can result in the formation of microcolonies. The cells can self-recognise and bind to the substrate by expressing surface adhesins (Trunk *et al.*, 2018).

Coaggregation of probiotic microorganisms with pathogens is essential in creating an unfavourable environment for oral pathogens, inhibiting pathogen overgrowth and proliferation, reducing pathogen growth and facilitating pathogen removal. Biofilm formation helps the pathogens to become more resistant to the host defence mechanism and antimicrobial compounds, thus the coaggregation of probiotic strains with pathogens creates a barrier that prevents biofilm formation (Matsubara *et al.*, 2016). In this study, *L. salivarius* could coaggregate with *S. mutans*, *S. sobrinus*, *C. albicans*, *C. glabrata* and *C. tropicalis*, while *L. casei* could only coaggregate with *S. mutans* and *S. sobrinus*. Probiotic bacteria interact closely with pathogens during this process, allowing them to release anti-pathogenic substances in close proximity to the pathogens. However, the combination of *L. casei* and *L. salivarius* did not work synergistically against oral pathogens in this study as no coaggregation was observed with *S. mutans*, *S. sobrinus*, *C. albicans*, *C. glabrata* and *C. tropicalis*.

The use of only a single time point (i.e., 4 h) in the aggregation assays in the present study could be a limitation of this study. More research should be done at various time points and stages of microbial growth to see whether there is a difference in the effect of aggregation abilities.

CONCLUSION

L. salivarius CFS alone demonstrated greater antimicrobial activity than *L. casei* alone or in combination. *L. salivarius* showed superior auto-aggregation and coaggregation abilities on its own. The combination of *L. salivarius* and *L. casei* did not work synergistically against selected oral pathogens because *L. salivarius* alone has a stronger antagonistic effect than the combination. To the best of our knowledge, this is the first study to report the antagonistic effect of *L. salivarius* and *L. casei* combination on selected oral pathogens. The association between *L. salivarius* adhesion, aggregation and cell surface properties should be investigated further to determine its possible probiotic use.

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