

IN VITRO ANTAGONISTIC GROWTH EFFECTS OF *LACTOBACILLUS FERMENTUM* AND *LACTOBACILLUS SALIVARIUS* AND THEIR FERMENTATIVE BROTH ON PERIODONTAL PATHOGENS

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ABSTRACT

As lactobacilli possess an antagonistic growth property, these bacteria may be beneficial as bioprotective agents for infection control. However, whether the antagonistic growth effects are attributed to the lactobacilli themselves or their fermentative broth remains unclear. The antagonistic growth effects of *Lactobacillus salivarius* and *Lactobacillus fermentum* as well as their fermentative broth were thus tested using both disc agar diffusion test and broth dilution method, and their effects on periodontal pathogens, including *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis in vitro* at different concentrations and for different time periods were also compared. Both *Lactobacillus salivarius* and *Lactobacillus fermentum* and their concentrated fermentative broth were shown to inhibit significantly the growth of *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis*, although different inhibitory effects were observed for different pathogens. The higher the counts of lactobacilli and the higher the folds of concentrated fermentative broth, the stronger the inhibitory effects are observed. The inhibitory effect is demonstrated to be dose-dependent. Moreover, for the lactobacilli themselves, *Lactobacillus fermentum* showed stronger inhibitory effects than *Lactobacillus salivarius*. However, the fermentative broth of *Lactobacillus fermentum* showed weaker inhibitory effects than that of *Lactobacillus salivarius*. These data suggested that lactobacilli and their fermentative broth exhibit antagonistic growth activity, and consumption of probiotics or their broth containing lactobacilli may benefit oral health.

Key words: *Lactobacillus fermentum*, *Lactobacillus salivarius*, *Streptococcus mutans*, *Streptococcus sanguis*, *Porphyromonas gingivalis*, periodontitis.

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INTRODUCTION

Periodontitis, one of the most prevalent oral diseases, is associated with the imbalance of indigenous microbiota (10), and subsequently induces overgrowth of periodontal pathogens including *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* (10, 21, 25, 27, 28). To treat periodontitis, antibiotic therapy is usually administered along with traditional treatments, including scaling and root planning, for reducing the bacteria and preventing the recurrence of infection (7, 12). However, some individuals with periodontal disease respond neither to the treatment of scaling and root planning alone nor to treatment in combination with antibiotic therapy (32). Moreover, antibiotic resistance poses further problems, thus limiting the application of antibiotic therapy in the treatment of periodontitis (30, 31). Therefore, additional strategies on the use of probiotics in prevention and treatment of periodontal disease are strongly recommended.

Lactobacilli colonize naturally in the vagina and digestive tract, and possess antagonistic growth properties that offer protection from invasive pathogens (20). Genitourinary infections lead to a shift in the local flora from a predominance of lactobacilli to coliform uropathogens. Use of lactobacillus-containing probiotics to restore commensal vaginal flora has been proposed for the treatment and prophylaxis of bacterial urogenital infections (6). Among lactobacilli genera, *Lactobacillus salivarius* and *Lactobacillus fermentum* are two of the most prevalent species in human saliva (9, 10). The genera belonging to this group can produce organic acids, such as lactic acid and acetic acid from carbohydrate fermentation, which can interfere with the growth of surrounding microorganisms and hydrogen peroxide which are antimicrobial substances (14). As lactobacilli possess an antagonistic growth property, these bacteria may be beneficial as bioprotective agents for infection control. Non-antibiotic therapy (25) has recently been applied in the treatment of periodontal diseases and has resolved the problem of antibiotic

resistance (30, 31). However, whether the antagonistic growth activity is attributed to the lactobacilli themselves or their fermentative broth remains unclear.

The aim of this study was to determine the antagonistic growth effects of *Lactobacillus salivarius* and *Lactobacillus fermentum* as well as their fermentative broth on growth inhibition of periodontal pathogens, including *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis*. In the present study, the antagonistic growth effects of these two species of lactobacilli and their fermentative broth were tested using both disc agar diffusion test and broth dilution method. In addition, their effects on the three well-known periodontal pathogens *in vitro* at different concentrations and for different durations were also compared.

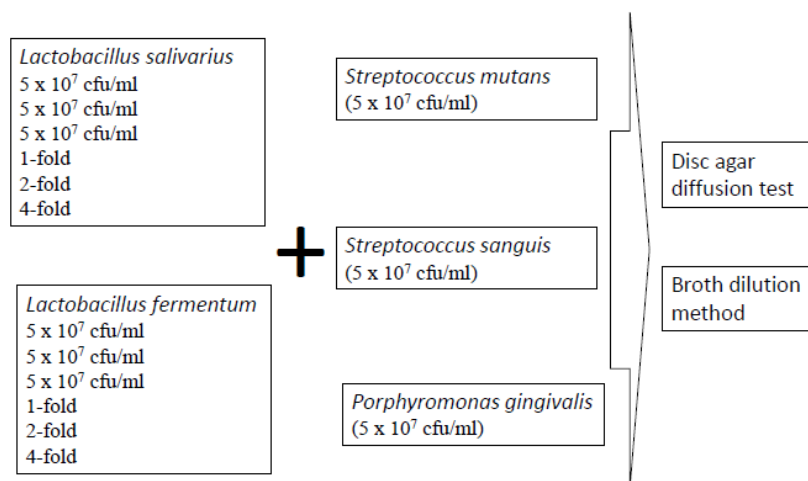
MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains, including *Streptococcus mutans* (ATCC25175), *Streptococcus sanguis* (ATCC49295), and *Porphyromonas gingivalis* (ATCC33277) were purchased from Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C.) and cultured according to the manufacturer's instructions. *Streptococcus mutans* was grown on tryptic soy broth (DIFCO 0369) with 5% defibrinated sheep blood at 37°C under aerobic conditions. *Streptococcus sanguis* was grown on brain heart infusion (BHI) broth (DIFCO 0418) at 37°C under aerobic conditions. *Porphyromonas gingivalis* was grown on BHI-T-C medium at 37°C under anaerobic conditions. In a screening of our collection of 22 species of lactobacilli, *Lactobacillus salivarius* and *Lactobacillus fermentum* were the best two lactobacilli that demonstrated antagonistic growth properties. Both lactobacillus species, *Lactobacillus fermentum* (strain SG-A95) and *Lactobacillus salivarius* (strain SG-M6), provided by Syngen Biotech Co. Ltd. (Tainan, Taiwan) were cultured in Mann Rogosa Sharp (MRS) broth at 37°C under

anaerobic conditions. 5×10^7 cfu of *Lactobacillus fermentum* and *Lactobacillus salivarius* were cultured in 20 ml MRS for 24 hours (hr) to prepare the fermented broth. The fermented broth of *Lactobacillus salivarius* and *Lactobacillus fermentum* were then centrifuged at 10,000 rpm for 10 minutes (min) to remove the cell pellet and concentrated by a rotary vacuum evaporator at 60°C as the concentrated fermentative broth. In

order to reduce the salts present in the concentrated fermentative broth, dialysis with a membrane (Spectra/Por®Dialysis Membrane; MWCO:3500, Spectrum Laboratories Inc, CA, USA) was performed for 48 hr before subsequent experiments. The fermentative broth and the lactobacilli themselves were stored at -80°C until required (Flowchart 1).



Flowchart 1. Summarizing the preparation steps involved in the present study.

Disc agar diffusion test

Paper discs infused with different concentrations of fermented MRS were placed on the surface of agar plates inoculated with the three different bacteria mentioned above, and incubated at 37°C for 24 hr in triplicate. Distilled water and 250 mg/mL of tetracycline were used as negative and positive controls, respectively. Each sample was tested by three repeated analyses. The diameter of the inhibition zone around the disc was measured (mm). The diameter of the inhibition zone for the negative control (dist. water) was 6 mm.

Broth dilution method

One milliliter of *Lactobacillus salivarius* or *Lactobacillus fermentum* suspension stock at counts of 5×10^7 cfu/ml, 5×10^8 cfu/ml or 5×10^9 cfu/ml; or 2-fold or 4-fold concentrated fermented broth of *Lactobacillus salivarius* or *Lactobacillus*

fermentum was co-cultured with 1 ml of *Streptococcus mutans*, *Streptococcus sanguis*, or *Porphyromonas gingivalis* at a count of 5×10^7 cfu/ml under periodontal bacteria's culture conditions as described above for different durations. The numbers of periodontal bacterial strains, i.e. *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis*, were counted at each time point and compared with unused MRS co-cultured with bacterial strains (negative controls). The number of bacterial strains was calculated by plating on agar plates as described above. Each sample was tested by three repeated analyses. The percentage of inhibitory was calculated as $100\% - [(Test\ group \div Control) \times 100\%]$.

Statistical analysis

Experimental results are presented as mean values. The Kruskal-Wallis test was employed to detect the difference in

in growth inhibition of periodontal pathogens among three or more than three groups, and Scheffé correction was performed to check statistically significant difference between groups. *P* value of less than 0.01 was considered significant. The data were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) statistical software.

RESULTS

Disc agar diffusion test

The growth inhibitory effects of the fermentative broth of

Lactobacillus fermentum and *Lactobacillus salivarius* on *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* were demonstrated by disc agar diffusion test. The inhibitory diameters of different concentrations of *Lactobacillus fermentum* and *Lactobacillus salivarius* fermentative broth were shown in Table 1. Significant inhibitory effects of the fermentative broth of both *Lactobacillus fermentum* and *Lactobacillus salivarius* at different concentrations were demonstrated except for that at 1-fold concentration.

Table 1. Growth inhibitory effects of fermentative broth of *Lactobacillus fermentum* and *Lactobacillus salivarius* on *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* obtained by disc agar diffusion test (n= 3).

Product	<i>Streptococcus mutans</i>	<i>Streptococcus sanguis</i>	<i>Porphyromonas gingivalis</i>
LFP 1-fold	6.0±0.0	6.0±0.0	6.2±0.2
LFP 2-fold	7.6±0.9	7.7±1.0	9.2±1.0
LFP 4-fold	13.2±1.2	12.4±1.2	9.7±0.6
LSP 1-fold	6.1±0.0	6.0±0.0	6.6±0.5
LSP 2-fold	9±1.0	9±1.0	12±1.0
LSP 4-fold	13±1.0	16±1.0	14±1.0
Tetracycline	29.7±2.0	25.9±1.2	33.4±2.2
Distilled water	6.0±0.0	6.0±0.0	6.0±0.0

LFP: *Lactobacillus fermentum* fermentative broth;
LSP: *Lactobacillus salivarius* fermentative broth

Broth dilution method

One milliliter of *Lactobacillus fermentum* or *Lactobacillus salivarius* suspension stock at counts of 5×10^7 cfu/ml, 5×10^8 cfu/ml, or 5×10^9 cfu/ml was co-cultured with 1 ml of 5×10^7 cfu/ml *Streptococcus mutans*, *Streptococcus sanguis*, or *Porphyromonas gingivalis* under these periodontal bacteria's culture conditions for different durations. Growth titers of *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* counted at different time points were used as negative controls for comparison (Fig. 1). The results are shown on Fig. 2. Significant growth inhibitory

effects ($p < 0.01$) were observed when lactobacilli were co-cultured with the pathogenic bacteria at a 1:1 ratio (5×10^7 cfu/ml). The higher the count (5×10^8 cfu/ml or 5×10^9 cfu/ml) of *Lactobacillus fermentum* or *Lactobacillus salivarius* treated, the higher the growth inhibitory effects ($p < 0.01$) on *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* were observed (Fig. 2). Moreover, lactobacilli at a higher ratio (100:1) exhibited stronger growth inhibitory effects on the three pathogenic bacteria tested than those at a lower ratio (10:1 or 1:1). For example, growth of *Streptococcus mutans* was completely inhibited after 48 hr of

1- (5×10^7 cfu/ml), 10- (5×10^8 cfu/ml) or 100-fold (5×10^9 cfu/ml) *Lactobacillus fermentum* treatment.

For *Streptococcus sanguis*, significant growth inhibition effects ($p < 0.01$) by 1- (5×10^7 cfu/ml), 10- (5×10^8 cfu/ml), and 100-fold (5×10^9 cfu/ml) of *Lactobacillus fermentum* or *Lactobacillus salivarius* treatment were also found (Fig. 2). For example, the growth of *Streptococcus sanguis* was completely inhibited after 14 hr of 1- and 10-fold or 10 hr of 100-fold *Lactobacillus fermentum* treatment, respectively.

For *Porphyromonas gingivalis*, when co-cultured with 5×10^7 cfu/ml, 5×10^8 cfu/ml or 5×10^9 cfu/ml *Lactobacillus fermentum* or *Lactobacillus salivarius*, significant growth inhibitory effects ($p < 0.01$) were also demonstrated (Fig. 2). For example, the growth of *Porphyromonas gingivalis* was completely inhibited after 14 hr of 1-fold, 8 hr of 10-fold or 6 hr of 100-fold *Lactobacillus fermentum* treatment, respectively. For the three oral pathogens tested, *Lactobacillus fermentum* showed stronger inhibitory effects than *Lactobacillus salivarius*.

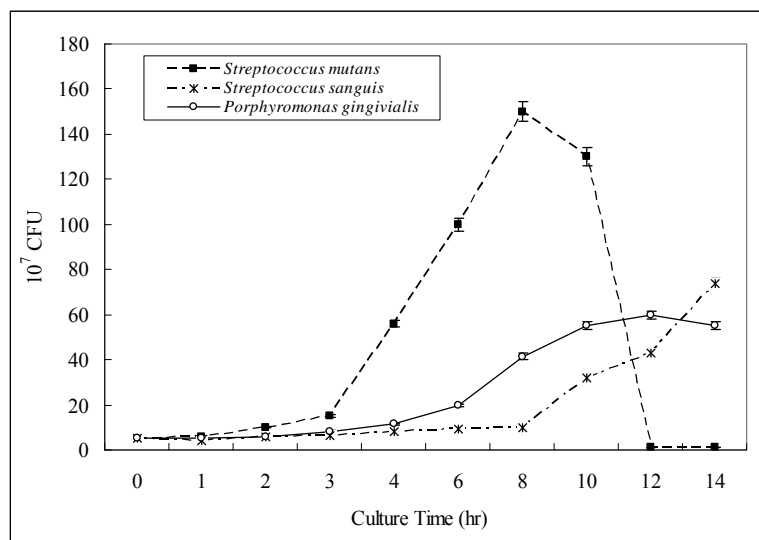
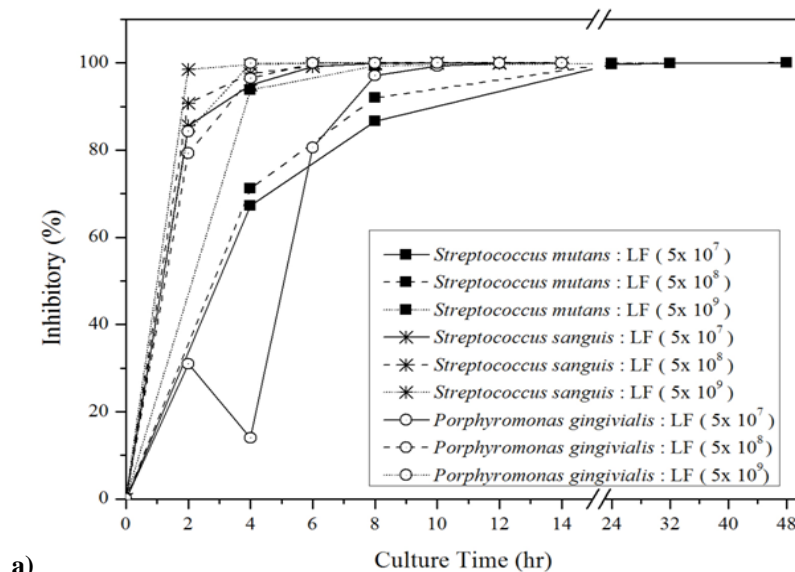


Figure 1. Growth curves of *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* tested at different time points and used as negative controls for test on inhibitory effects (n= 3).



a)

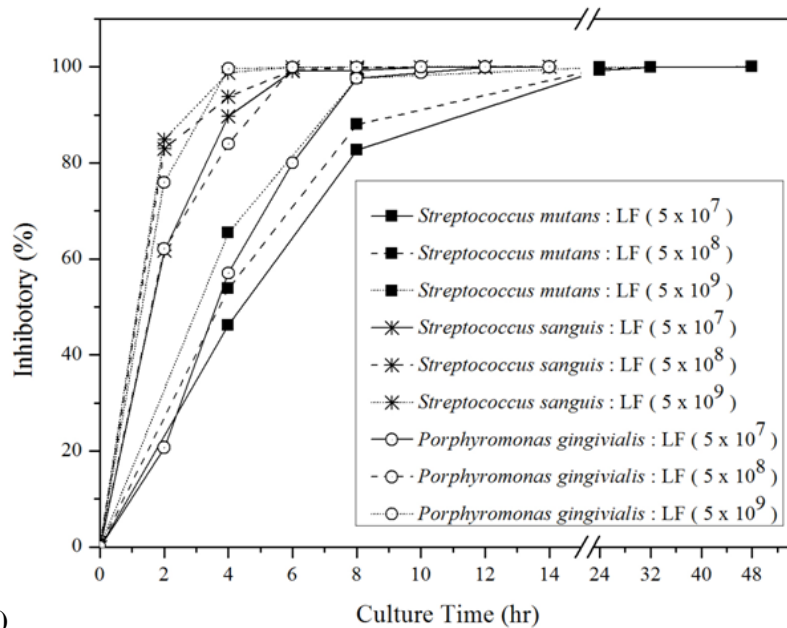
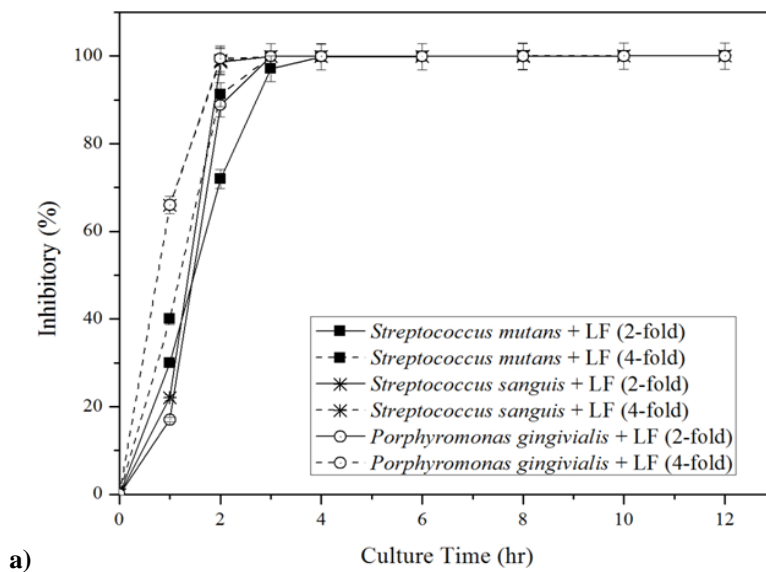


Figure 2. Growth inhibitory percentages of different counts of (a) *Lactobacillus fermentum* or (b) *Lactobacillus salivarius* on 5×10^7 cfu/ml *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* at different time points (n= 3).

Since 1-fold fermentative broth of *Lactobacillus fermentum* and *Lactobacillus salivarius* did not show significant inhibitory effects by disc agar diffusion test, 2- and 4-fold concentrated fermentative broth were thus used for test. The broth dilution method demonstrated significant growth inhibitory effects of the concentrated fermentative broth of *Lactobacillus fermentum* and *Lactobacillus salivarius* on periodontal pathogens (Fig. 3). Both 2-

and 4-fold concentrated fermentative broth of *Lactobacillus fermentum* and *Lactobacillus salivarius* inhibited significantly the growth of periodontal pathogens, including *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis*. However, the fermentative broth of *Lactobacillus fermentum* showed weaker inhibitory effects than that of *Lactobacillus salivarius*.



a)

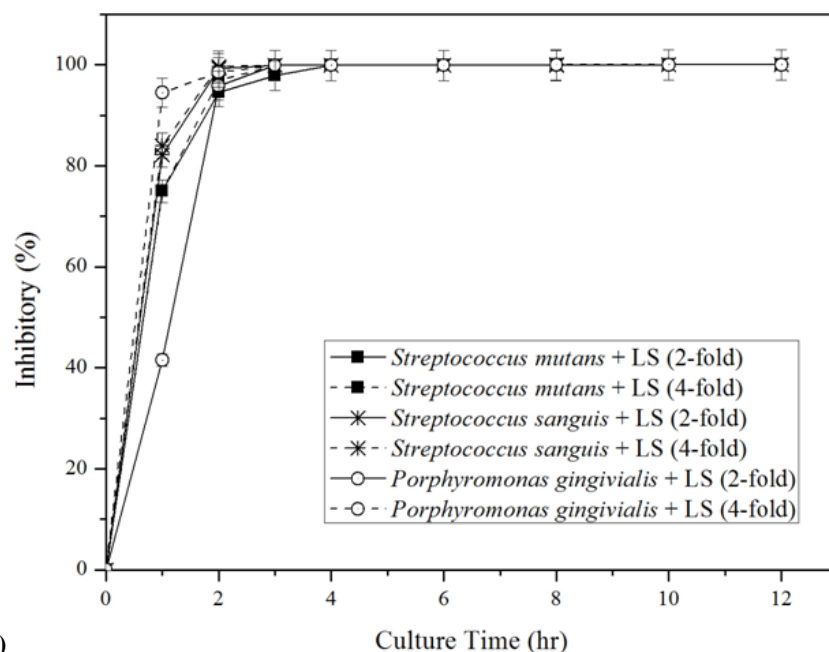


Figure 3. Growth inhibitory effect of different folds of concentrated (a) *Lactobacillus fermentum* or (b) *Lactobacillus salivarius* fermentative broth on 5×10^7 cfu/ml *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* at different time points (n= 3).

DISCUSSION

Probiotics are defined as microorganisms that generally confer a health benefit on humans (5, 8). Lactobacilli, which have been consumed daily by millions of people around the world for perceived health benefits, have been regarded as safe. Intestinal lactobacilli have been successfully used as probiotics to treat gastrointestinal disorders, but only limited data on the probiotic properties of oral lactobacilli for combating oral diseases are available. The use of probiotics in clinical trials should be accompanied by knowledge of the antagonistic growth susceptibilities of the organism used (24). In the present study, lactobacilli and their fermentative broth were tested *in vitro* for their potential probiotic properties for oral health. Both *Lactobacillus salivarius* and *Lactobacillus fermentum* and their fermentative broth were shown to inhibit the growth of three periodontal pathogens, i.e., *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis*, although different inhibitory effects were observed for different

pathogens. Moreover, the higher the counts of lactobacilli and the higher the folds of concentrated fermentative broth, the stronger the inhibitory effects were, indicating that the inhibitory effect was dose-dependent.

In the lactobacilli genera, it was reported that strains of *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* expressed high antimicrobial activity (11). *Lactobacillus salivarius* CECT5713 has recently been shown not only to be the best for *in vitro* antibacterial activity, but has also been found to possess the highest protective effect against a *Salmonella* strain in the murine infection model (19). *Lactobacillus salivarius* CELA2 (a bacteriocin-producing strain) was shown to display the highest probiotic potential in the gastrointestinal tract (13). *Lactobacillus fermentum* strain L23 and *Lactobacillus rhamnosus* strain L60 were suggested for prevention and treatment of urogenital infections in women, in view of their probiotic properties and production of bacteriocins (23). Short-term consumption of cheese containing

Lactobacillus rhamnosus GG and *Lactobacillus rhamnosus* LC705 has been shown to reduce the risk of *Streptococcus mutans* in young adults, although their long-term effects remain unclear (1). Application of *Lactobacillus reuteri* was found to result in reduced gum bleeding and gingivitis. Oral lactobacilli flora has also been shown to inhibit the growth of *Porphyromonas gingivalis* and *Prevotella intermedia* (10). Taken together, our data are consistent with other findings indicating that lactobacilli and their fermentative broth exhibit antagonistic growth activity and consumption of probiotics or their broth containing lactobacilli can prevent or treat periodontal disease (2, 17, 18). These results suggest a potential for the two lactobacilli species and their fermented broths to be used as probiotics and functional products, respectively for oral health.

Moreover, for the lactobacilli themselves, *Lactobacillus fermentum* showed stronger inhibitory effects than *Lactobacillus salivarius*. However, the fermentative broth of *Lactobacillus fermentum* showed weaker inhibitory effects than that of *Lactobacillus salivarius*. The mechanisms of probiotic action in the mouth are supposed to be similar to that observed in gastrointestinal tracts (15). Nevertheless, data on oral probiotics are yet insufficient. Probiotics has been used as passive local immunization vehicles to improve oral immune response and to prevent against oral diseases (2, 29). Recent reports on the search of antimicrobial protein/compounds produced by lactic acid bacteria have been increasing (4, 16, 22, 26). Furthermore, probiotics produce organic acids including lactic, acetic and formic acids, which lower pH and oxidation-reduction potential and may suppress harmful organisms. It is possible that the mechanism of action is attributed to the organic acids produced by the probiotics and present in the fermentative broth. However, in view of the difference in antibacterial potential between the two lactobacilli and their fermentative broth, such possibility was rather low. It was thus suggested the two lactobacilli species themselves may undergo different mechanisms to exhibit inhibitory effects on oral pathogens, e.g. the lactobacilli may

compete with oral pathogens for growth nutrients or growth space, which is different from the replication inhibition underwent by the antimicrobial protein/compounds in the fermentative broth. Further investigation is needed to elucidate this assumption.

In conclusion, we have demonstrated through *in vitro* growth inhibitory test that *Lactobacillus fermentum* and *Lactobacillus salivarius* and their concentrated fermentative broth inhibit significantly periodontal pathogens, including *Streptococcus mutans*, *Streptococcus sanguis*, and *Porphyromonas gingivalis* in a dose-dependent manner. Consumption of probiotics or their broth containing lactobacilli may benefit oral health. However, further and extensive researches including human studies are needed.

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