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Original Article

Increasing salivary IgA and reducing *Streptococcus mutans* by probiotic *Lactobacillus paracasei* SD1: A double-blind, randomized, controlled study

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Abstract *Background/purpose:* Our previous study revealed that probiotic *Lactobacillus paracasei* SD1 could reduce mutans streptococci as evaluated by cultivation-method as well as stimulate innate immunity. This study aimed to further investigate the effect of the probiotic on various oral bacteria by real-time PCR and salivary IgA levels.
Materials and methods: Forty children were included by randomization from either probiotic or control group in the previous study. The probiotic or control received milk-powder with or without *L. paracasei* SD1, respectively once daily for 6 months. Saliva were collected at baseline 3-, 6-, and 12-months and were evaluated for total bacteria, total lactobacilli (TL), *L. paracasei/L. casei* (LP/LC), total streptococci (TS) and *Streptococcus mutans* using the real-time PCR. The salivary IgA (sIgA) was examined using the ELISA method.
Results: All target bacterial levels were not significantly different at baseline in both groups. After milk-powder consumption, TL and LP/LC levels were significantly increased in the probiotic group, whereas TS and *S. mutans* levels were significantly decreased compared to baseline. TS and *S. mutans* levels were significantly lower, while the sIgA was greater in the probiotic compared to the control group. In the probiotic group, a positive correlation was found between LP/LC and sIgA, while negative correlations were observed between TS or *S. mutans* levels and sIgA.
Conclusion: L. paracasei SD1 could control *S. mutans* level and could stimulate sIgA. Results indicate that the *L. paracasei* SD1 strain may have a benefit for prevention of dental caries.

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Introduction

Over the past decades, probiotics, especially *Lactobacillus* strains, have proved to prevent and treat several intestinal and systemic diseases.[1] After ingestion, probiotics would integrate to the native bacterial microflora leading to improving its homeostasis.[2] Probiotics could compete with pathogens for colonization, stimulate local and systemic immunities, and reduce the inflammatory response.[1,3,4]

Recently, an increased interest in probiotics for oral health has emerged, and the concept of caries prevention by probiotics has been proposed.[5–8] Our previous studies explored the effect of *Lactobacillus paracasei* SD1 on prevention of caries and its safety. It revealed that either short-term or long-term consumption of milk-powder containing *L. paracasei* SD1 could reduce salivary cariogenic mutans streptococci resulting in low caries risk.[7–10] Those studies used a selective agar, modified mitis salivarius bacitracin agar, to distinguish *Streptococcus mutans* and *Streptococcus sobrinus* as a mutans streptococci group from other streptococci; however, it may not clearly distinguish those two species from each other.[11] Other limitations of the cultivation method such as low sensitivity and variation in growth conditions call for using other methods to confirm previous results.[12,13] Molecular methods are more appropriate for bacterial detection due to better sensitivity and specificity, less time consuming, and reducing laboratory-labor. Real-time PCR has been recently recommended because of its sensitive and rapid method for detection and quantification of bacteria.[13] It is based on the detection of 16s rRNA of bacteria, which presents in multiple copies in all bacterial species and contains highly conserved species-specific sequences.

In addition, our previous study demonstrated that probiotic *L. paracasei* SD1 could stimulate human neutrophil peptides (HNP) 1–3, the first line of host defense against microorganisms, in saliva. The HNP1-3 level was found significantly higher in the probiotic group than in the control group.[14] Several studies also reported that probiotics could increase immunoglobulin A (IgA) after probiotic administrations.[15,16] Secretory IgA is the immunoglobulin found in all secretions which has its main role in saliva to aggregate and block the binding of microorganisms to mucosal membrane and teeth.[17,18] Therefore, it is interesting to study the effect of *L. paracasei* SD1 intervention on stimulating salivary IgA (sIgA).

The aim of this study was to monitor the effects of *L. paracasei* SD1 on the growth of mutans streptococci as well as other bacteria using real-time PCR. The sIgA level was also determined after probiotic consumption using ELISA.

Materials and methods

Subjects and saliva collection

Unstimulated whole saliva samples derived from our previous study, originally from a double-blinded, randomized placebo-controlled trial, were used. The study design, test group, participants and sample collection were described earlier.[8] The study was re-approved by the Faculty of Dentistry Ethics Committee at the Prince of Songkla University, Thailand for the continued study.

Subjects included 40 volunteers (22 females and 18 males), aged 12–14 years old (mean: 13.6 ± 0.5 years). They were divided equally into two groups using a random technique with a placebo group (12 females and 8 males) and probiotic group (10 females and 10 males). There was no significant difference between the groups.

Salivary samples of individual volunteers who consumed milk-powder with or without probiotic *L. paracasei* SD1 5g/day for six months was collected at baseline (T0), 3 (T3), 6 (T6), and 12 (T12) months, and was then divided into two equal volumes for examination of microbial and sIgA levels. If the salivary samples were not assayed directly, they were frozen at −80 °C until further processing.

Real-time PCR quantification

DNA from the salivary samples (50 μl) were extracted using a Genomic DNA Extraction Kit (RBC Bioscience, Taipei, Taiwan) following the manufacturer's protocol for salivary bacteria, and the DNAs were kept at −20° until used.

The quantities of total bacteria and targeting bacteria in saliva (at T0, T3, T6, and T12) were evaluated using real-time PCR. Total bacterial DNA (5 μl) was added in a SensiFAST™SYBR kit (Bioline Reagent Ltd., California, USA). The sequences of primer used were as follows: total bacteria (5′-TCCTACGGGAGGCAGCAGT-3′ and 5′-GGACTACCAGGGTATCTAATCCTGTT-3′),[19] total lactobacilli (5′-CATTTGGAAACAGATGCTAATACC-3′ and 5′-GTCCATTGTGGAAGATTCCC-3′) (this study), total streptococci (5′-CCGGTGACGGCAAGCTAA-3′ and 5′-TCATGGAGGCGAGTTGCA-3′),[20] *Lactobacillus paracsei/L. casei* (5′-AATACATGCAAAGTCGACGAG-3′) and (5′- TTGGATCTATGCGGTATTAGCA-3′) (this study), *S. mutans* (5′-ACTACACTTTCGGGTGGCTTGG-3′ and 5′-CAGTATAAGCGC CAGTTTCATC-3′)[21] and *S. sobrinus* (5′-GATAACTACCTGACAGCTGACT-3′ and 5′-AAGCTGCCTTAAGGTAATCACT-3′).[21] The PCR thermal profile consisted of an initial DNA step of 10 min at 95 °C followed by 2 min at 50 °C and 40 cycles of 95 °C for 20 s with different annealing temperatures including 60 °C for total bacteria and *S. mutans*, 58 °C for total lactobacilli, *L. paracasei/Lactobacillus casei* and

S. sobrinus, and 56 °C for total streptococci for 20 s and the polymerizing temperature at 72 °C for 25 s. Amplification, detection, and data analysis were performed with CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, USA). Each sample was run in triplicate. A standard curve was plotted for each targeting bacteria with cycle threshold (CT) values obtained from amplification of known quantities of bacteria (log CFU/ml) as follows: *Escherichia coli* ATCC25922, *Lactobacillus fermentum* ATCC14931, *Streptococcus sanguinis* ATCC10556, *L. paracasei* CCUG32212, *S. mutans* ATCC25175, and *S. sobrinus* ATCC33478 for total bacteria, total lactobacilli, total streptococci, *L. paracasei*/*L. casei*, *S. mutans*, and *S. sobrinus*, respectively. A standard curve was constructed using the CT values from the qPCR and presented as log CFU/ml.

Determination of salivary immunoglobulin A (sIgA) using an ELISA assay

The sIgA was quantified using an indirect sandwich enzyme-linked immunosorbent assay (ELISA).[18] The plates were coated with goat anti-human α-chain specific IgA overnight. The coated plates were washed with a washing buffer (PBS pH 7.0 contained 0.05% Tween-20) and blocked by 1% BSA for 1 h at 37 °C in a moist chamber. Saliva samples were diluted at 1:500 with a washing buffer containing 0.5% BSA and then 100 μl of diluted saliva was added into the coated plate. The plate was incubated at 37 °C for 1 h 30 min in a moist chamber. Human serum containing monomeric human IgA was served as a reference standard (15.62—1000 ng). An amount of 100-μl of mouse anti-human IgA, the primary antibody, was added to each well. The plate was incubated at 37 °C for 1 h in a moist chamber and then it was washed with the washing buffer. Then the secondary antibody (goat anti-mouse IgA conjugate with horseradish peroxidase) was added to each well. The plate was incubated at 37 °C for 1 h in a moist chamber. After washing, enzymatic activity was stimulated by o-phenylenediamine and 0.03% H_2O_2 in 0.1 M citrate-phosphate buffer, pH 5.0. The reaction was stopped by the addition of 100 μl of 2 N H_2SO_4. The absorbance was determined at OD 490 nm using a microplate reader. sIgA concentration was compared with the total protein concentration of an individual sample, which was performed according to Bradford's assay.[22] All tests were carried out in duplicate.

Statistical analysis

The data were expressed as means ± standard deviation (SD). The comparative differences of bacterial numbers and sIgA between groups in individual time were evaluated using the independent sample-T test. The changes in the bacterial numbers and sIgA individual time in the same group were analyzed using the Bonferroni test. Correlations of total lactobacilli, total streptococci, *S. mutans* and sIgA in each group were tested using Pearson's Correlation test. $P < 0.05$ was considered statistically significant.

Results

A number of various bacteria in the salivary samples

Genomic DNA extracted from various bacteria and tested for the specificity of primers using PCR is shown in Fig. 1. The formulation derived from the standard curves of each target bacteria was used to analyze the bacterial number showed as log CFU/ml.

This study monitored the microbial community including total bacteria, total lactobacilli, total streptococci, *L. paracasei*/*L. casei* (LP/LC), *S. mutans*, and *S. sobrinus* in salivary samples as shown in Figs. 2 and 3. Total bacteria in both groups were not significantly different throughout the study either within or between groups (Fig. 2A, $P > 0.05$). Total lactobacilli and LP/LC levels of children in the probiotic group significantly increased after receiving probiotic milk-powder (at 3-, 6- and 12 months) compared to the baseline (Fig. 2B, C, $P < 0.05$), while this was not observe in the control group. The levels of LP/LC in both groups at baseline (3.6 ± 0.5 and 3.4 ± 0.6 log CFU/ml) was not a statistically significant difference (Fig. 2C, $P > 0.05$), nonetheless the LP/LC level at 3-, 6-, and 12- months in the probiotic group was found to be higher than in the control group (Fig. 2C, $P \leq 0.001$).

Total streptococci in the probiotic and control groups were not significantly different at baseline (with means 7.9 ± 0.6 and 7.8 ± 0.5 log CFU/ml, respectively). After 3 months receiving probiotic milk-powder; total streptococci in the probiotic group showed to be significantly lower than in the control group at T3 (7.2 ± 0.7 and 8.4 ± 0.6 log CFU/ml), T6 (7.2 ± 0.6 and 8.4 ± 0.7 log CFU/ml), and T12 (7.4 ± 1.6 and 8.5 ± 0.7 log CFU/ml), which all gave

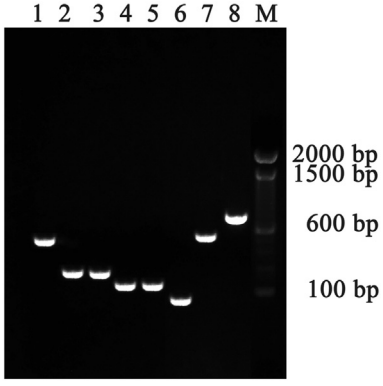

Figure 1 The specific primer of this study was tested by PCR. Lanes: 1, *Escherichia coli* ATCC25922 with primer for total bacteria; 2—3, *Lactobacillus paracasei* CCUG32212 and *L. fermentum* ATCC14931 with primer for total lactobacilli; 4—5, *L. paracasei* CCUG32212 and *L. casei* ATCC393 with primer for *L. paracasei*/*L. casei*; 6, *Streptococcus sanguinis* ATCC10556 with primer for total streptococci; 7, *S. mutans* ATCC25175 with primer for *S. mutans*; and 8, *S. sobrinus* ATCC33478 with primer for *S. sobrinus*.

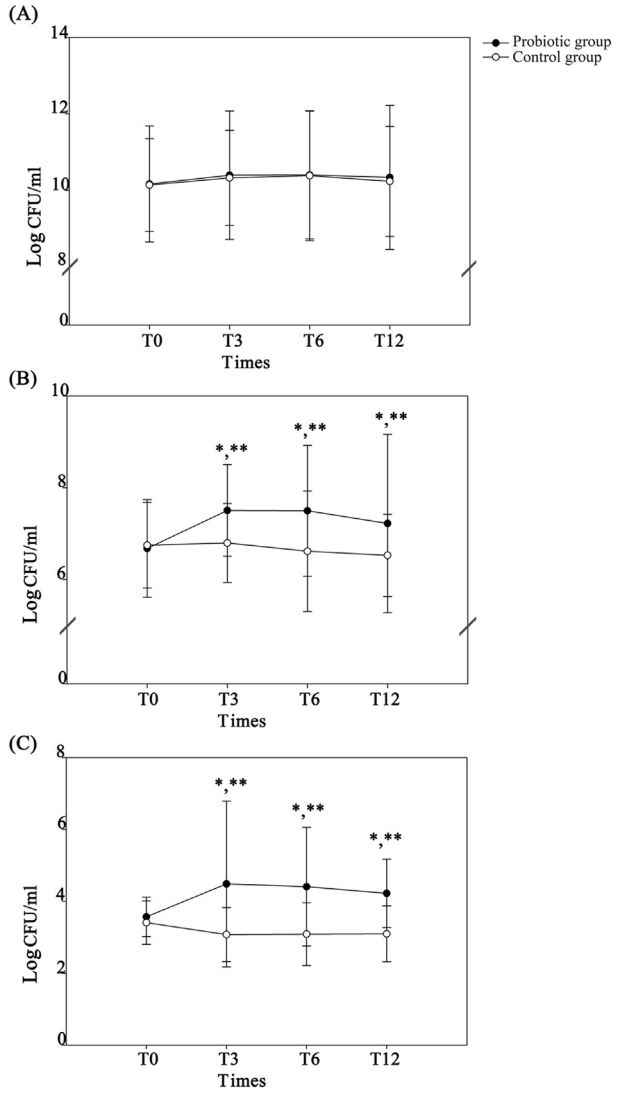

Figure 2 The number of bacteria at the baseline (T0) and during the 3-, 6-, 12- months of study (T3—T12) by (A) Total bacteria; (B) Total lactobacilli; (C) *L. paracasei*/*L. casei*. * significant difference between the probiotic group and control group was evaluated by an independent sample t-test; the parameters were evaluated by the Bonferroni test **significant difference between T3, T6, and T12 and T0 in the probiotic group.

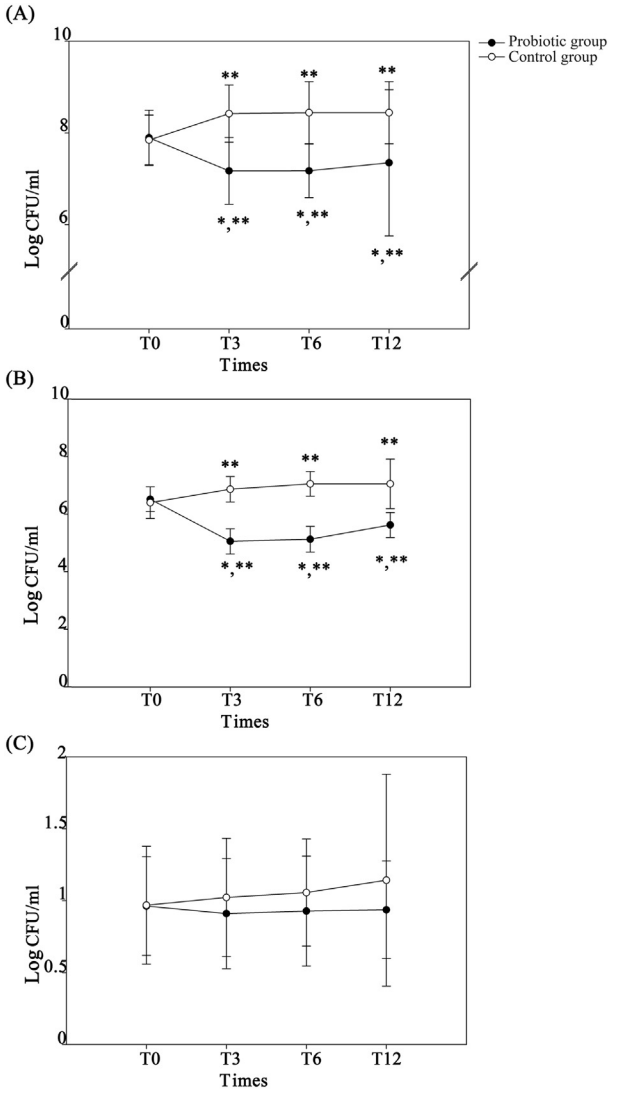

Figure 3 The number of bacteria at the baseline (T0) and 3-, 6-, 12- months of study (T3—T12); (A) Total streptococci; (B) *Streptococcus mutans*; (C) *S. sobrinus*. * significant difference between the probiotic group and control group was evaluated by an independent sample t-test; the parameters were evaluated by the Bonferroni test where **significant difference was found between T0 and T3 or T6 or T12 in the probiotic group and the control group.

statistical differences at $P < 0.001$ (Fig. 3A). Total number of streptococci in the control group was significantly increased at 3-, 6- and 12-months compared to the baseline (Fig. 3A: at $P = 0.040$, $P = 0.043$, and $P = 0.024$, respectively), while it was significantly decreased in the probiotic group at 3-, 6- and 12 months compared to the baseline (Fig. 3A: at $P = 0.002$, $P = 0.002$, and $P = 0.001$, respectively).

S. mutans levels at baseline of the probiotic and control groups (means 6.5 ± 1.2 and 6.4 ± 1.6 log CFU/ml, respectively) were not statistically different (Fig. 2B, $P > 0.05$), however, they were found significantly higher in the control compared to the probiotic group at 3-, 6-, and 12- months (Fig. 3B, $P = 0.007$, $P = 0.025$, and $P = 0.035$, respectively). *S. mutans* levels in the probiotic group significantly decreased at 3-, 6-, and 12- months compared to the baseline (Fig. 3B: at $P = 0.009$, $P = 0.011$, and $P = 0.031$, respectively), whereas the levels was significantly increased in the control group at 3-, 6- and 12- months compared to the baseline (at $P = 0.043$, $P = 0.043$, and $P = 0.050$, respectively).

S. sobrinus levels were not significantly different at the baseline in both groups and they were not significantly different throughout the study in both groups compared to the baseline (Fig. 3C, $P > 0.05$).

sIgA level

The sIgA levels of the control and probiotic groups were not significantly different ($P > 0.05$) at the baseline. After

receiving the probiotic milk-powder, the sIgA level in the probiotic group significantly increased at 3-, 6- and 12-months compared to the baseline (Fig. 4: $P = 0.000$, $P = 0.000$, and $P = 0.003$, respectively) and it was also higher than the control group at 3-, 6- and 12- months (Fig. 4: $P = 0.000$, $P = 0.015$ and $P = 0.017$, respectively).

Correlation between sIgA and bacterial levels

A significant positive correlation was found between the sIgA level and LP/LC in the probiotic group (Fig. 5A: $P = 0.045$, $r = 0.260$), but it was not observed in the control group (data not shown). An increase of sIgA showed a significantly negative correlation with total streptococci and the *S. mutans* level in the probiotic group (Fig. 5B: $P = 0.042$, $r = -0.374$ and Fig. 5C: $P = 0.038$, $r = -0.380$).

Discussion

Monitoring the effects of *L. paracasei* SD1 on the growth of mutans streptococci as well as other bacteria or bacterial groups examined in this study confirmed that the real-time PCR method showed a higher sensitivity and specificity than the cultivation method used in the previous study.[8] The results of the real-time PCR examination displayed significantly higher level of lactobacilli (mean 7.0 ± 0.4 log CFU/ml) and mutans streptococci (mean 6.2 ± 0.3 log CFU/ml) compared to the same bacteria and the same samples were analyzed by the cultivation method in our previous results (means 6.3 ± 1.1 and 3.9 ± 1.3 log CFU/ml, for lactobacilli and mutans streptococci, respectively).[8] Using the real-time PCR showed an approximate 10—58% increase.

In addition, the present study aimed to monitor the change in levels of target bacteria *S. mutans*, *S. sobrinus* and *L. paracasei*/*L. casei* after receiving the probiotic *L. paracasei* SD1 strain. This study was not able to design specific primers for *L. paracasei* SD1 due to the close relation between the *L. paracasei* and *L. casei* strains and was therefore not specifically identified in samples after the probiotic administration period, but probably reported

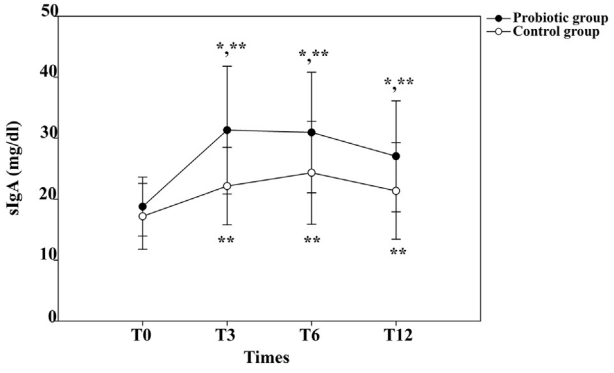

Figure 4 The quality of salivary IgA at the baseline (T0) and 3-, 6-, 12- months of study (T3—T12) *significant difference between the probiotic group and control group was evaluated by an independent sample t-test; the parameters were evaluated by the Bonferroni test **significant difference between T0 and others in the probiotic group and the control group.

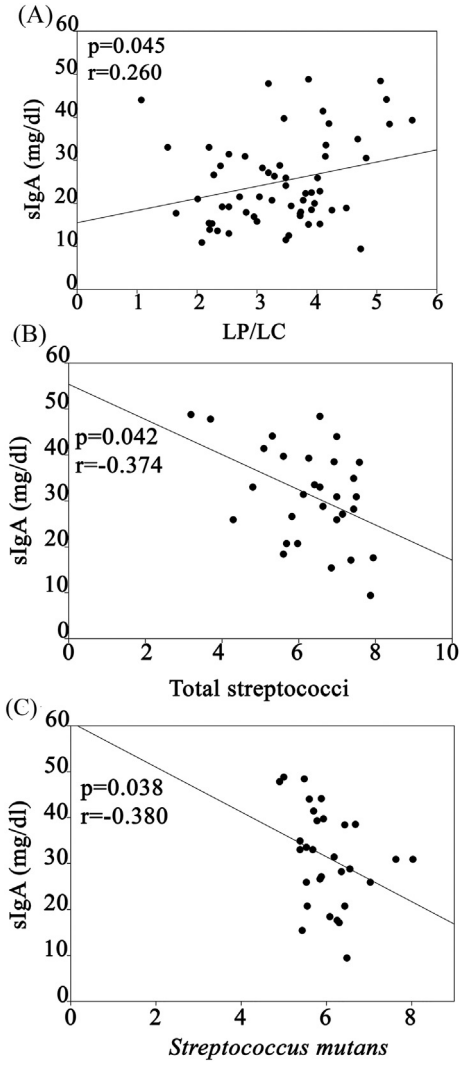

Figure 5 Positive and negative correlations between salivary IgA (sIgA) levels and *Lactobacillus paracasei*/*L. casei* (LP/LC) as well as between total streptococci or *Streptococcus mutans* and sIgA, in the probiotic group. The correlation coefficients (r) and the significance levels (*P*-values) between each pair of four parameters, including sIgA levels, LP/LC, total streptococci, and *S. mutans* were determined and illustrated for the probiotic groups (A—C).

in the group of *L. paracasei*/*L. casei* if present. In addition, the real-time PCR could separately specify the *S. mutans* and *S. sobrinus* levels. Results indicated that the real-time PCR could detect microorganisms at the species level by using individual specific primers, generally based on bacterial 16s rRNA containing highly conserved species-specific sequences, is rapid and has a high accuracy compared to cultivation method.[13,23] Although, the real-time PCR is relatively expensive and requires special apparatus and the potential risk to overestimate bacterial levels due to amplification of DNA non-viable bacterial cells. The real-time PCR technique is a preferable method for bacterial detection in analysis of target bacteria. It suggests that the choice for choosing the method used depends on facilities available in each laboratory.

After receiving probiotic milk-powder, the levels of total lactobacilli and *L. paracasei*/*L. casei* were significantly increased while total streptococci and *S. mutans* were significantly decreased, compared to the control group. This indicates that such findings resulted from receiving the probiotic *L. paracasei* SD1 due to a positive correlation between lactobacilli and *L. paracasei*/*L. casei* ($P = 0.001$, $r = 0.517$, data not shown). Also, our previous study could detect approximately 85% *L. paracasei* SD1 during the intervention period (3 months of receiving probiotic milk-powder).[8] A decrease of total streptococci and *S. mutans* in this study is in agreement with the reduction of the mutans streptococci level in the previous study.[8] A number of studies including a systemic review have shown that the presence of mutans streptococci, especially *S. mutans*, in saliva or plaque of 2- to 5-year old children is correlated with an increased risk of dental caries.[8,10,24] Besides, other species clearly arise as main players in the microbial community including *Veillonella*, *Rothia*, and *Leptotrichia* in enamel caries and *S. sanguinis*, *Atopobium*, *Schlegelella*, *Pseudoramibacter* and *Lactobacillus* in dentine caries.[25] However, mutans streptococci are mostly chosen to be the representative cariogenic organism for monitoring the caries risk.[10,26,27] The *S. sobrinus* level did not change throughout the study in either group, which may be due to the low prevalence of *S. sobrinus* in general.[28,29] This suggests that determination of the *S. mutans* level in saliva is sufficient to monitor the change in the cariogenic microbiota.

It was noticed that total bacterial levels were not significantly different in either group compared to the baseline, while levels of certain organisms differed after probiotic consumption. This may imply that probiotic strains may maintain the balance of microorganisms as a whole, resulting in no difference to the total bacteria levels.[30,31]

An increase of sIgA was found among volunteers both in the probiotic and control groups, however, the level of this immunoglobulin was significantly higher in the probiotic than in the control group after receiving milk-powder containing the probiotic *L. paracasei* SD1 strain. A positive correlation between sIgA and total lactobacilli was found. This indicates that *L. paracasei* SD1 may exert an immune-stimulatory effect by enhancing the sIgA production. This is in agreement with a previous study, in which probiotics could improve the gut mucosal or oral immune system by increasing the number of sIgA in the intestine or oral cavity.[32] However, sIgA also was found to slightly increased in the control group, this may be due to the stimulation by the skim milk. It has been reported that sIgA in bronchoalveolar lavage fluid of mice increased after receiving skim milk.[33] Results from the present and the previous study[14] suggest that *L. paracasei* SD1 could enhance both sIgA and innate immune HNP 1–3 response. In this study, a negative correlation was found between sIgA and *S. mutans*/total streptococci levels, that may result from the prevention of colonization by host immunity (sIgA and innate immune HNP 1–3). Although it was not the purpose of this study, the association between the gender and oral bacterial levels and sIgA was also analyzed. No any significant association was found.

In conclusion, real-time PCR is a method that gives more sensitivity and specificity for monitoring the level of microflora. This study intends to monitor the effect of the probiotic strain at pre-, during- and post-intervention on oral bacteria and sIgA. Results clearly revealed that *L. paracasei* SD1 could reduce *S. mutans* levels and enhance sIgA indicating that the *L. paracasei* SD1 strain may have a benefit for prevention of dental caries.

Conflicts of interest

The authors declare no potential conflict of interest with respect to the authorship and/or publication of this article.

Acknowledgments

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