Original Article

Inhibition of *Streptococcus mutans* by a commercial yogurt drink

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Abstract *Background/Purpose:* Studies have been focused on using probiotics to prevent caries. The lactobacillus probiotic bacteria in Yakult® (LcY) has been shown to inhibit the growth or biofilm formation of *Streptococcus mutans*. However, sucrose in Yakult® raised concerns. The purpose of this study was to determine effects of Yakult® on the growth and adhesion of *S. mutans*.
Materials and methods: *S. mutans* was grown in serial diluted Yakult®, filtered Yakult® or 20% heated Yakult®. *S. mutans* was co-cultured with LcY in media with or without diluted filtered Yakult®, or in LcY grown in media with or without sugars. Colony forming units and pH values of bacterial cultures were determined. SYTO 9-stained adhered bacteria were observed.
Results: Yakult® inhibited the growth of *S. mutans*. Filtering or heating Yakult® reduced its inhibitory ability against *S. mutans*. The inhibitory effect of LcY against *S. mutans* was enhanced when cultured in the presence of 20% filtered Yakult®. LcY cultured in sucrose media for 24 h inhibited the growth of *S. mutans*, but this effect was less evident when LcY was grown for 48 h. LcY grown in glucose or lactose media similarly reduced *S. mutans* growth. Culturing *S. mutans* with LcY grown in sucrose or glucose media reduced bacterial adhesion. However, co-culturing *S. mutans* with LcY grown in the lactose media did not decrease bacterial adhesion.
Conclusion: Yakult® and its probiotic content may inhibit *S. mutans* growth and the effect may be moderated by the type of sugar added for LcY cultivation.

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Introduction

Probiotics, defined by the WHO/FAO guidelines, are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host".[1] Commercialized probiotics are usually strains belonging to the genus of *Lactobacillus*, *Bifidobacterium*, *Enterococcus* or *Streptococcus*.[2] Traditionally, researches have been focused on health benefits of probiotics to the intestinal tract. Since oral infectious diseases, such as caries and periodontal diseases, are caused by dysbiosis, extending the application of probiotics to maintain or reform a healthy oral microflora is considered as a potential method for prevention or treatment of oral diseases. Recent studies provide controversial but encouraging evidences for the therapeutic potential of probiotics against oral diseases, especially caries, as indicated by systematic reviews.[3,4] Meta-analyses show that, while caries incidence is not significantly changed after probiotic consumption, probiotics reduce the level of *Streptococcus mutans*, the cariogenic pathogen, in the oral cavity.[3,4]

Probiotics not only decreased the amount of oral *S. mutans in vivo*. *In vitro* studies have shown that co-culturing *S. mutans* with certain probiotic strains,[5–8] or culturing *S. mutans* in cell-free culture medium of probiotics,[9,10] such as *Lactobacillus acidophilus* (DSM 13241[6]), *Lactobacillus casei* (ATCC SD5213,[6] ATCC 393,[10] LPC37, Shirota[8]), *Lactobacillus kefiranofaciens* (DD2[5]), *Lactobacillus paracasei* (LMG-P-17806,[6] LPC37[8]), *Lactobacillus plantarum* (curd isolates,[9] ATCC 14917,[10] ST-III[8]), *Lactobacillus reuteri* (ATCC 23272[10]), *Lactobacillus rhamnosus* (LGG,[7] LR32[6]), *Lactobacillus salivarius* (clinical isolates,[7] ATCC 11741[10]), *Bifidobacterium animalis* subsp. *lactis* (DSM 15954[6]), reduced the growth of *S. mutans*. In addition, some studies showed that co-culturing *S. mutans* with probiotics or culturing *S. mutans* in cell-free media of probiotics reduced its biofilm formation.[6,10–12]

Among previous clinical studies, the most common vehicles for probiotic delivering were dairy foods, such as milk, ice cream or fermented milk products.[4] Tablets, lozenges, candies, gum, drinks or liquids, powders, straws, cereals or toothpastes containing probiotics were also used.[4,13] To enhance the flavor of these probiotic carriers, sugars are usually added during their production. Sweetening of these probiotic-containing products brings concerns about their cariogenic potential. However, an *in situ* study shows that the presence of bactericidal molecules, such as antibiotics, may protect demineralization of dental tissues, which is exposed to sucrose and then put into the oral cavity of volunteers.[14] Thus, even under cariogenic conditions, it is suggested that bactericidal factors which influence dental microbes may protect dental tissues from infection. On the other hand, the carbohydrate source and the nutrient availability are reported to affect the antagonistic capacity of commensal or probiotic bacteria that are able to inhibit the growth or biofilm formation of *S. mutans*.[15,16] When abundant studies[5–7,9,10] determine the effects of probiotic isolates against *S. mutans*, with few using dairy products,[17,18] it is difficult to evaluate whether sugars or other constituents in a product would compromise the inhibitory effect of the probiotic strains.

Yogurt drinks are reported to be the third most popular snack among Taiwanese preschool children.[19] These drinks usually contain dominant amounts of sugars, which are added for fermentation and taste. For example, a bottle of Yakult®, the first commercialized and one of the most popular yogurt drink in Taiwan, contains 13.6 g added sugars, as claimed by the manufacturer. According to the information provided by Yakult® Australia, the most abundant sugar in Yakult® is sucrose.[20] While sucrose is shown to reduce the susceptibility of *S. mutans* toward antimicrobial agents,[21] the sucrose-containing Yakult® is reported to reduce salivary *S. mutans* in children whose salivary *S. mutans* level at baseline is higher than 10^5 colony forming units (CFU)/ml.[22] In contrast, three other studies report that salivary *S. mutans* levels are not significantly changed after consumption of Yakult®.[23–25] When the probiotic strain, *L. casei* strain Shirota, is shown to inhibit the growth of *S. mutans*,[8,12] the controversial results of clinical studies lead to a question: Could this sweetened probiotic beverage inhibit the viability of *S. mutans*? Thus, in this study, effects of Yakult® on the growth of *S. mutans* were investigated. Factors in its content that may affect the antagonizing capacity of Yakult®, such as its probiotic strain or sugar sources, were also evaluated.

Materials and methods

Bacterial cultivation

S. mutans ATCC 25175 was cultured in Bacto™ brain heart infusion (BHI; BD Bioscience, San Jose, CA, USA) at 37 °C in an atmospheric environment. Bacterial culture was streaked on a BHI agar plate (1.5% agar) and grown at 37 °C for 24 h before use. The bacterial strain in a bottle of Yakult® (Yakult® Co., Ltd., Taipei, Taiwan) was isolated by streaking an aliquot of fermented Yakult® on a BHI agar plate. Several single colonies were inoculated and grown in the BHI broth at 37 °C in an atmospheric environment. DNA was extracted from bacterial cultures and subjected to the polymerase chain reaction (PCR) using the universal primers 27F and 1492R.[26] The amplicon was sequenced by the High-Throughput Sequencing Platform at National Yang-Ming University VYM Genome Research Center (Taipei, Taiwan). The data were aligned with the gene sequence of the *L. casei* strain isolated from the Yakult® product (LcY)[27] using

the NCBI Nucleotide BLAST. The sequence similarity of the amplicon to the reference was 99%.

Culturing of S. mutans in Yakult®

Yakult® or Yakult® that was filtered through a 0.22 μm filter (Startorius Stedim Biotech, Aubagne Cedex, France) was serially diluted by the BHI broth. In certain experiments, Yakult® was heated at 55 °C or 65 °C for 30 min and then diluted to 20% before use. In certain experiments, levels of LcY in diluted or heated Yakult® were determined by streaking serial diluted solution on BHI agar plates and calculating colony forming units (CFU) of LcY. A single colony of S. mutans was inoculated in the BHI broth and grown at 37 °C for 24 h. A 100-fold dilution of the S. mutans suspension with 3 ml of BHI without or with heated or diluted Yakult® yielded a solution containing approximately 10^5 CFU/ml. S. mutans was then incubated at 37 °C for 15 h, serial diluted and streaked on BHI agar plates. Since it took one or three days for colonies of S. mutans or LcY, respectively, to be seen on the plate (data not shown), colonies of S. mutans were calculated at 24 h post inoculation and converted to log[CFU/ml] for data analyses. The pH values of Yakult®, filtered Yakult® and the bacterial cultures after incubation were measured by a pH meter (pH/ORP Meter SP-2300; SUNTEX, New Taipei City, Taiwan). Filtering did not affect the pH of Yakult®, which was 3.62 ± 0.03 (n = 9).

Culturing of S. mutans with LcY

In experiments involving co-culturing S. mutans and LcY, a single colony of LcY was inoculated and cultured in BHI broth or 20% filtered Yakult® at 37 °C for 3 days to the stationary phase. After being washed with the culture medium, cells were counted using the C-Chip disposable hemocytometer (DHC-N01; NanoEnTek, Seoul, Korea). S. mutans (10^5 CFU/ml) was then cultured without or with various concentrations of LcY. Bacterial cultures were incubated at 37 °C for 15 h, serially diluted and streaked on BHI agar plates. Colonies of S. mutans were counted at 24 h post-inoculation and converted to log[CFU/ml].

In some conditions, S. mutans was co-cultured with LcY in BHI broth containing 100 mM of sucrose, glucose or lactose. LcY was grown for 3 days from a single colony into the stationary phase. LcY suspension was then added into the BHI broth containing 100 mM sucrose, glucose or lactose in 1:100 dilutions, which yielded bacterial suspensions containing LcY of approximately 10^7 CFU/ml. LcY was further cultured in media containing sucrose, glucose or lactose at 37 °C for 24 or 48 h. S. mutans was then added to 3 ml of LcY suspensions, to a final concentration of approximately 10^5 CFU/ml. Bacteria were cultured at 37 °C for 15 h, serially diluted, streaked on BHI agar plates and incubated for 1 day or 3 days for colony calculation of S. mutans and LcY, respectively.

Adhesion assay

The levels of adhered S. mutans on coverslips were quantified as previously reported[28] with modifications. LcY (10^7 CFU/ml) was incubated in the BHI broth containing 100 mM of sucrose, glucose or lactose, and cultured for 24 h in 24-well culture plates, in which 12-mm coverslips were placed. S. mutans was then added to a final concentration of 10^5 CFU/ml and further cultured for 15 h at 37 °C. After washed, bacteria on coverslips were stained with SYTO 9 (Life Technologies, Carlsbad, CA, USA) and then fixed in 4% paraformaldehyde. Samples were mounted in the fluorescent mounting medium (DAKO, Glostrup, Denmark) and observed by a fluorescent microscope (DM IRB; Leica, Solms, Germany). At least 3 images (600 ×) of each sample were captured and saved as RGB files using the software Metamorph (version 7.8.0.0; Molecular Devices, LLC., Sunnyvale, CA, USA). Numbers of fluorescent particles were defined and quantified by the software ImageJ (NIH, Bethesda, MD, USA). The value obtained from the particle counts of S. mutans grown in the sucrose medium without LcY was set as 100%, to normalize the particle counts of other groups.

Statistical analyses

The results obtained were analyzed by one-way analysis of variance (one-way ANOVA), using the software Statistical Product and Service Solutions (SPSS, version 20; IBM, Armonk, NY, USA). Tukey test was used for post-hoc analyses. Statistic differences were set at the significant level of $\alpha = 0.05$.

Results

Yakult® inhibited the growth of S. mutans

To determine the effects of Yakult® on the growth of S. mutans, S. mutans (10^5 CFU/ml) in the stationary phase was cultured in serially diluted Yakult®. As a mixture made from fermentation of skimmed milk and sugars by the L. casei strain Shirota, factors such as probiobiotic lactobacilli or organic acids might contribute to its effects on S. mutans. To differentiate which proportion was involved in its inhibitory effect, Yakult® was also filtered through 0.22 μm filters and serially diluted for the growth of S. mutans. Filtered Yakult® was clear, suggesting filtering removed both the probiotic lactobacilli and colloidal contents in Yakult®. S. mutans was also cultured in the BHI broth (0% Yakult®) for comparison. Colony forming units of S. mutans and pH of bacterial cultures were both determined. After cultured in the BHI broth for 15 h, S. mutans was grown to $1.5 \times 10^{13} \pm 5.0 \times 10^{12}$ CFU/ml (n = 4). When cultured in serially diluted Yakult®, the number of live S. mutans in the medium was decreased in a concentration dependent manner (Fig. 1A, solid rhombi). Growing of S. mutans in 40% and 80% Yakult® significantly reduced the amount of live bacteria. Yakult® diluted to 80% by the BHI broth almost depleted live S. mutans (3.3 ± 2.3 CFU/ml; n = 4). The inhibitory effect on the growth of S. mutans was not observed, when filtered Yakult® diluted to 40% or further diluted to a less concentration (Fig. 1A, empty rhombi). However, live S. mutans was not detectable, when grown in filtered Yakult® diluted to 80% (n = 4). The pH values of the S. mutans cultures grown in BHI for 15 h were 7.26 in

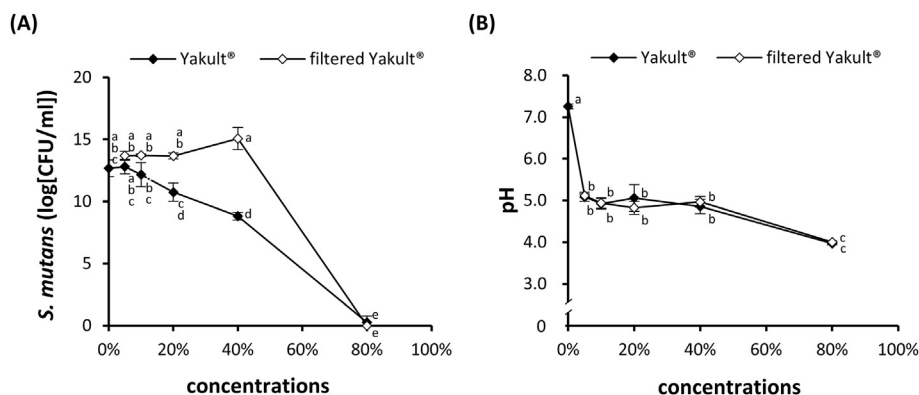**Figure 1** Effects of Yakult® on the growth of planktonic *S. mutans*. *S. mutans* was added into serial diluted Yakult® (filled rhombi) or filtered Yakult® (empty rhombi) to final concentrations of 10^5 CFU/ml, and grown at 37 °C for 15 h. (A) Bacterial samples were then serial diluted and streaked on BHI agar plates. After growing for 24 h, colonies of *S. mutans* were calculated and converted to log[CFU/ml]. (B) Bacterial samples were also collected for pH measurement. Results are shown as means ± standard errors of the mean (SE) from four independent experiments. Two samples were significantly different if indicated with completely different lower case letters (one-way ANOVA and Tukey test; $\alpha = 0.05$).

average (standard error = 0.08). The pH values of *S. mutans* planktonic cultures grown in Yakult® diluted to 5−40% were significantly reduced to about 5, and further decreased to 4 when 80% Yakult® was used (Fig. 1B). Changes in pH values of bacterial cultures grown in filtered Yakult® were not significantly different from those of bacterial cultures grown in Yakult® (Fig. 1B).

Data suggested that contents removed by filtering, i.e. probiotic lactobacilli and colloidal contents, contribute to the growth inhibition of *S. mutans* by Yakult®. To determine whether the inhibitory effect of Yakult® was heat-labile, Yakult® was not heated or heated at 50 °C or 65 °C and then diluted to 20% for the culture of *S. mutans*. In these experiments, *S. mutans* grown in 20% filtered Yakult® served as controls. These heating temperatures were chosen because the level of lactobacilli in Yakult® (LcY) which were heated at 50 °C and then diluted to 20% were not statistically different from 20% unheated Yakult®, while the level of lactobacilli in Yakult®, heated at 65 °C and then

diluted to 20%, was significantly reduced (Fig. 2A). Heated Yakult® was diluted to 20% based on two observations: (1) The level of *S. mutans* that were grown in 20% Yakult® was significantly less than the level of *S. mutans* in 20% filtered Yakult® (Fig. 1A); (2) pH values of Yakult® diluted to 40% or 80% were lower than 6 (data not shown). As shown in Fig. 2B, 20% unheated Yakult® or 20% Yakult® that had been heated at 50 °C inhibited the growth of *S. mutans*, when compared to the level of *S. mutans* grown in 20% filtered Yakult®. In contrast, the growth of *S. mutans* in 20% Yakult® that had been heated at 65 °C was not statistically different from bacterial growth in 20% filtered Yakult®.

LcY contributed to the inhibitory effects of Yakult® against *S. mutans*

Whether the probiotic strain in Yakult® was responsible to the inhibitory effect of Yakult® on *S. mutans* was further tested. According to the manufacturer's description, a
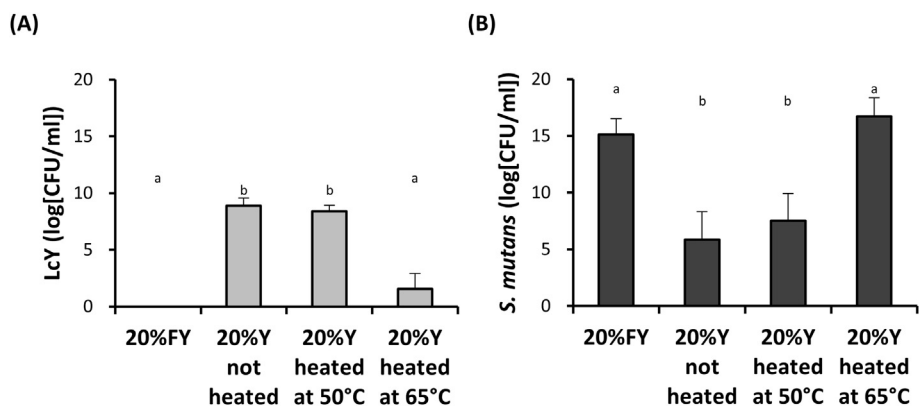

Figure 2 Effects of heating on the inhibitory ability of Yakult® against the growth of planktonic *S. mutans*. Yakult® (Y) was not heated or heated at 50 °C or 65 °C or filtered (FY) and then diluted to 20%. (A) Levels of LcY in various Yakult® samples were determined and shown as log[CFU/ml]. (B) *S. mutans* (10^5 CFU/ml) was cultured in 20% unheated, heated or filtered Yakult® for 24 h and then subjected to CFU determination. Results are shown as means ± SE from four independent experiments. Two samples were significantly different if indicated with different lower case letters (one-way ANOVA and Tukey test; $\alpha = 0.05$).

bottle of Yakult® sold in Taiwan contains at least 10^8 cells/ml of probiotic lactobacilli. We also found that, Yakult® that was diluted to 80% by the BHI broth contained $6.1 \times 10^8 \pm 2.1 \times 10^8$ CFU/ml of live bacteria (n = 4). Thus, the probiotic lactobacillus strain in Yakult® (LcY) was adjusted to $5 \times 10^7 - 8 \times 10^8$ cells/ml and co-cultured with *S. mutans* in the BHI broth. Considering that fermentation of LcY might affected its inhibitory effect against *S. mutans*, LcY was also co-cultured with *S. mutans* in 20% filtered Yakult® containing sugars that were added for fermentation and taste. Compared to *S. mutans* grown in the BHI broth, co-culturing with LcY significantly reduced the amount of live *S. mutans* (Fig. 3A, white bars). The level of live *S. mutans* in the group that used 8×10^8 cells/ml of LcY in BHI was $0.002 \pm 0.001\%$ of the control group. However, *S. mutans* was not eradicated ($1.3 \times 10^6 \pm 7.3 \times 10^5$ CFU/ml), when LcY of 8×10^8 cells/ml was present. This finding was different from the result obtained from culturing *S. mutans* in 80% Yakult, which depleted live *S. mutans* (Fig. 1A). When *S. mutans* was co-cultured with LcY in 20% filtered Yakult®
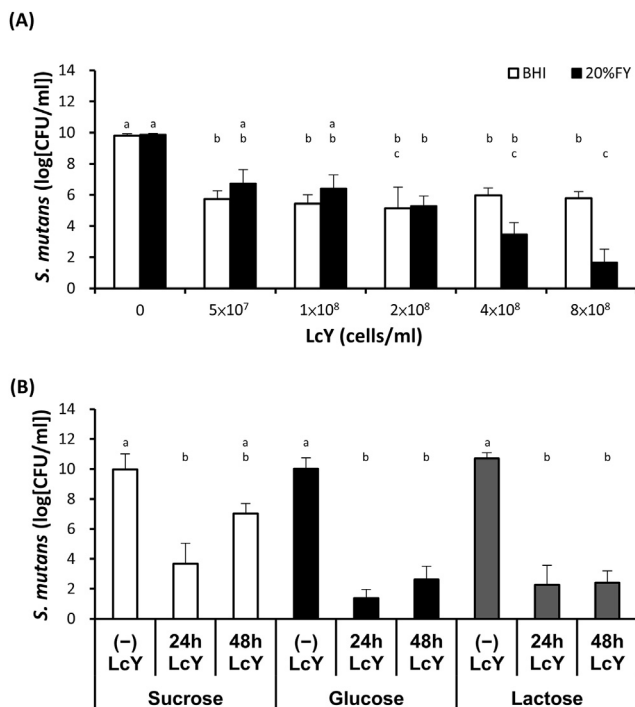
(Fig. 3A, black bars), LcY reduced the growth of planktonic *S. mutans* in a concentration dependent manner. Compared to the groups that grew *S. mutans* and LcY in the BHI broth, when co-cultured with 8×10^8 cells/ml of LcY in 20% filtered Yakult®, live *S. mutans* was significantly decreased (Fig. 3A, white and black bars).

According to the information provided by Yakult® Australia, a bottle of Yakult® contains 15.7% (460 mM) sucrose, which constitutes almost 90% sugars in Yakult®.[20] Thus, to test whether the fermentation of sucrose affected the inhibitory ability of LcY against *S. mutans*, *S. mutans* was co-cultured with LcY that had been grown in the presence of 100 mM sucrose, the level of which might be close to the level of sucrose in 20% filtered Yakult®. Two other sugars in Yakult®, glucose and lactose,[20] were also added in the medium to grow LcY for comparison. The levels of live LcY grown in sucrose media for 24 or 48 h were not statistically different (Table 1). In contrast, pH values of LcY cultures were higher when growing in the sucrose medium, compared to those growing in the glucose or lactose medium (Table 1). Compared to the group that grew *S. mutans* in the BHI broth containing sucrose (the white bar labeled with (−) LcY), the number of live *S. mutans* was decreased by cultures of LcY that was grown in sucrose media for 24 h, but not for 48 h (Fig. 3B, white bars). Interestingly, LcY grown in glucose or lactose media for 24 or 48 h reduced the amount of live *S. mutans* significantly (Fig. 3B, black and grey bars).

LcY affected the adhesion of bacteria

The effects of LcY on adhesion of *S. mutans* were further determined. Co-culturing *S. mutans* with LcY grown in the sucrose medium effectively reduced the amount of bacterial aggregates on the coverslips (Fig. 4A), which were 8% of the level of attached *S. mutans* grown in the absence of LcY (Fig. 4B). When bacterial particles in the group co-culturing *S. mutans* with LcY grown in the glucose medium were dense and bright, particles in the group co-culturing *S. mutans* with LcY grown in the lactose medium were lighter (Fig. 4A). The level of adhered *S. mutans* that were cultured in the glucose or lactose medium was $113 \pm 26\%$ and $55 \pm 10\%$, respectively, compared to *S. mutans* cultured in the sucrose medium (Fig. 4B). Co-culturing *S. mutans* with LcY in the glucose medium resulted in dispersed small

(A)

(B)

Figure 3 Effects of LcY on the growth of planktonic *S. mutans*. (A) *S. mutans* (10^5 CFU/ml) was grown in the BHI broth (BHI) or 20% filtered Yakult® (20%FY) without or with various concentrations of LcY ($5 \times 10^7 - 8 \times 10^8$ cells/ml) at 37 °C for 15 h. (B) LcY was inoculated into the BHI broth containing 100 mM of sucrose, glucose or lactose, and then cultured at 37 °C for 24 h (24 h LcY) or 48 h (48 h LcY). *S. mutans* (10^5 CFU/ml) was then cultured in LcY suspension or the BHI broth containing each carbohydrate ((−) LcY) and grown at 37 °C for 15 h. Bacterial samples were then serially diluted, streaked on BHI agar plates and grown for 24 h for CFU determination. Results are shown as means ± SE (n = 3) of planktonic *S. mutans* expressed as log(CFU/ml). Two samples were significantly different if indicated with completely different lower case letters (one-way ANOVA and Tukey test; $\alpha = 0.05$).

Table 1 Levels of live LcY and medium pH values of BHI broth containing 100 mM sucrose, glucose or lactose after growing LcY for 24 or 48 h (n = 4).

	Sucrose	Glucose	Lactose
Live LcY (log[CFU/ml])			
24 h	9.38 ± 0.11^a	9.13 ± 0.22^a	9.40 ± 0.15^a
48 h	9.23 ± 0.12^a	9.04 ± 0.28^a	9.05 ± 0.20^a
pH values			
24 h	5.15 ± 0.05^a	4.25 ± 0.05^{bc}	4.35 ± 0.05^{bc}
48 h	4.60 ± 0.10^b	3.90 ± 0.00^c	4.05 ± 0.15^c

Note: Results are shown as means ± SE. Two samples were significantly different if indicated with completely different lower case letters (one-way ANOVA and Tukey test; $\alpha = 0.05$).
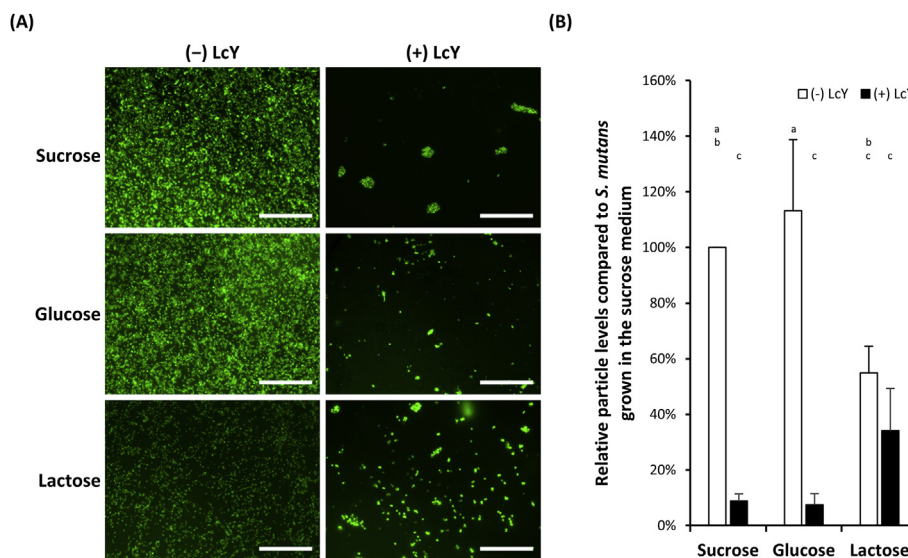
Figure 4 Effects of different carbohydrates on inhibition of bacterial adhesion by LcY. LcY in the stationary phase was cultured in BHI broth containing 100 mM of sucrose, glucose or lactose at 37 °C for 24 h ((+) LcY). Media without LcY ((−) LcY) were also cultured and used as controls. *S. mutans* was then added to a final concentration of 10^5 CFU/ml, and further cultured at 37 °C for 24 h to form biofilms. Bacterial biofilms were stained with SYTO 9 and then observed using a fluorescent microscope. The representative images (600×) of four independent experiments are shown. The white bars in the images represent 50 μm. (B) Bacterial particles in each treatment were quantified. The value obtained from *S. mutans* grown in the sucrose medium without LcY was set as 100% to normalize particle counts of other groups. Results are shown as means ± SE (n = 4) of percentage of particles levels. Two samples were significantly different if indicated with completely different lower case letters (one-way ANOVA and Tukey test; $\alpha = 0.05$).

particles on the coverslips. The level of adhered bacteria that were cultured in the glucose medium was similar to the level of *S. mutans* cultured in the sucrose medium (Fig. 4B). More small and brighter bacterial aggregates were observed on coverslips collected from the group that co-cultured *S. mutans* and LcY in the lactose medium, compared to the group that co-cultured *S. mutans* with LcY in the sucrose medium (Fig. 4A). The amount of attached bacteria in the lactose medium in the absence or presence of LcY was not statistically different (Fig. 4B).

Discussion

Regarding whether Yakult® reduced salivary levels of *S. mutans*, clinical studies showed controversial results.[22−25] The present study showed that Yakult® could inhibit the growth of *S. mutans*. The probiotic content of Yakult® contributed to this antimicrobial effect, since filtering and heating reduced the ability of Yakult® to inhibit *S. mutans* growth (Figs. 1 and 2). This finding echoed with a clinical study showing that daily mouth-rinsing with Yakult® for 7 constitutive days reduced salivary levels of *S. mutans*.[22] While three other studies did not show reduction of salivary levels of *S. mutans* after Yakult® consumption,[23−25] the reason for the inconsistent observation is still unclear. A potential explanation is that the study designs of these clinical studies varied and might not be standardized among studies, as suggested by systemic reviews.[3,4] Additionally, as the data showed, types of sugars added in the culture media or the growing status affected the inhibitory efficiency of the probiotic bacteria against *S. mutans* (Fig. 3B). Although

Yakult® is distributed worldwide, some local manufacturers modify raw materials for its production, potentially leading to differences in the inhibitory ability of Yakult®.

Sugar contents in Yakult® and other yogurt drinks always raise concerns. In contrast to the non-sweetened milk, cheese or yogurt, a bottle of 100 ml Yakult® contains 13.6 g (about 0.9 tablespoons) added sugars, as claimed by the manufacturer. Added sugars for moderately physically active children who are less than 8 years old should be limited to 3 tablespoons (about 3 bottles of Yakult®) per week, as the Dietary Approaches to Stop Hypertension (DASH) diet plan suggests.[29] On the other hand, culturing LcY in sugar-containing media decreased the pH values to less than 5.5 (Table 1), a result of carbohydrate fermentation and organic acid production. Organic acids are shown to be responsible for antimicrobial activities of probiotics.[30] Considering that the optimal pH for F-ATPase, the enzyme maintaining the intracellular homeostasis of pH in *S. mutans*, is 6,[31] and that glycolysis activities of *S. mutans* are inhibited by extracellular pH less than 5 in a dose dependent manner,[32] the acidified culture medium should inhibit the activity of *S. mutans*. This is also supported by the finding that inhibitory efficiency was inversely related to the medium pH values: the sucrose medium after LcY culturing was less acidified than the lactose or glucose medium (Table 1), while the level of *S. mutans* cultured with LcY grown in sucrose medium for 48 h tended to be higher than the ones co-cultured with LcY in glucose or lactose medium (Fig. 3B). However, the pH values of sucrose media after LcY grown for 24 h were higher than 48 h (Table 1), while the growth of *S. mutans* was inhibited more evidently by LcY cultured in sucrose media for 24 h,

compared to the 48-h cultures (Fig. 3B). Therefore, data suggested that, while organic acids played a role in the inhibition of *S. mutans* growth, other factors may also be involved. The mechanism behind this observation remains to be examined.

Although a poor surface colonizer,[33] LcY reduced bacterial adhesion and this effect also depended on sugar additives in the culture media. LcY grown in the glucose or sucrose medium, but not the lactose one, reduced adhesion of *S. mutans* significantly (Fig. 4). *Lactobacilli* can affect biofilm formation of *S. mutans* via interfering genes that are related to biofilm formation[7,34] or co-aggregation that masks the receptors for binding on tooth surfaces.[35] Treatment of LcY with various carbohydrates may alter these properties, and thus, affects the formation of biofilms. A previous study showed that simultaneously co-culturing *S. mutans* with LcY in the presence of 0.2% sucrose (5.8 mM) tended to increase the gene expression of glucosyltransferases, which are responsible for the production of extracellular polysaccharides (EPS) and cell-cell-interaction during *S. mutans* biofilm formation.[33] This finding seems to contradict to the result of this study. However, in addition to that the concentrations of sucrose are different between these two studies (100 mM vs 5.8 mM), the timing for bacterial co-culturing was also different. In this research, *S. mutans* was added after LcY was grown in sucrose media for 24 h, pH values of which were reduced to about 5. In the previous study,[33] *S. mutans* and LcY were simultaneously added into the sucrose media, the pH values of which were neutral. Thus, bacteria in these two studies might respond differently, due to changes in osmotic or acid stress.

When viability of *S. mutans* grown in the medium containing lactose was not significantly different from *S. mutans* grown in medium containing glucose or sucrose (Fig. 3), adhesion of *S. mutans* was reduced compared to the other two groups (Fig. 4). This finding can be explained by a study showing less biomass, soluble proteins or insoluble EPS in the *S. mutans* biofilm grown in the lactose medium.[36] The reduction of EPS may be related to the absence of dextran-associated EPS, which are produced by *S. mutans* cultured in the lactose medium.[37]

In this study, effects of Yakult® or LcY on the growth of *S. mutans* and bacterial adhesion were determined. When the bactericidal effect of Yakult® depends on its acidity, the potential of enamel demineralization by this fermented milk product should be considered. An *in vitro* study showed that commercial yogurt drinks, including Yakult®, decreased surface microhardness of enamel blocks. Reduction of enamel surface microhardness was inversely related to the concentrations of calcium in the yogurt drinks.[38] Enamel wear was shown to be restricted in superficial surfaces.[38] *In situ* studies compared plaque acidification and enamel demineralization in patients consumed commercial yogurt drinks or 20% sucrose by forming biofilms on bovine enamel slabs, which were placed in the oral cavity of human subjects.[24,39] The pH values of dental plaque[39] and enamel demineralization[24,39] were not different between subjects taking Yakult® or sucrose. In contrast, another brand of yogurt drink increased pH values of dental plaque and reduced enamel demineralization.[24,39] This observation is different from a study showing that short-term Yakult®

consumption reduced acid production in dental plaque.[23] Differences among these studies might be a result of differences in their designs and manufacturers; additionally, the anti-cariogenic effects of yogurt drinks might be varied between brands.

In conclusion, our results support an inhibitory effect of Yakult® and its probiotics, LcY, on the growth of *S. mutans*. Although sugars in this drink raise concerns in health issues, acidity, a product of carbohydrate fermentation by LcY, likely contributes to the inhibitory effect of Yakult® against *S. mutans*. The type of sugars in the culture media may modulate the ability of LcY against *S. mutans*. Considering that concerns related to sweetening of the beverage and involvement of carbohydrate fermentation in the inhibitory effect of LcY, further studies may be focused on searching formula that counterbalances both issues.

Conflicts of interest

None declared.

Acknowledgement

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