Original Article

pISSN, eISSN 0125-5614
M Dent J 2025; 45 (3) : 248-265

Effect of low-sugar fermented milk with *Lactobacillus paracasei* 431 on *Streptococcus mutans* biofilm formation and enamel demineralization: *in vitro* study

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Objectives: This study investigates the effects of *Lactobacillus paracasei* 431 and sugar content in fermented milk on the growth and biofilm formation of *S. mutans*, as well as its potential to cause enamel demineralization.
Materials and Methods: Two fermented milks were used: 1.) 6% sucrose, and 2.) 0.1% sucrose. *S. mutans* (ATCC25175) was cultured with fermented milk and filtered fermented milk to remove *L. paracasei* 431. Firstly, *S. mutans* growth was determined by using colony-forming units. Secondly, *S. mutans* biofilm formation was evaluated using a biofilm assay. Thirdly, *S. mutans* biofilms were grown on enamel slabs, then exposed twice per day to the fermented milks for five days, and surface hardness loss was measured. Biofilms on the enamel slabs were stained with live/dead dye and observed under a confocal microscope. The Kruskal-Wallis test was used to compare differences among groups, and post-hoc test contrasts for every pair of variables were used (Bonferroni Correction). A difference was considered statistically significant if it was $p<0.05$.
Results: The viable *S. mutans* count in fermented milk with 6% sucrose was lower than in filter-sterilized milk ($p<0.05$). Both fermented milks with 6% sucrose and 0.1% sucrose reduced *S. mutans* biofilm formation compared to filter fermented milk ($p<0.05$). Fermented milk with 0.1% sucrose showed less demineralization than 6% sucrose, though the difference was not significant ($p>0.05$).
Conclusions: Fermented milk with *Lactobacillus paracasei* 431 inhibited *S. mutans* growth and biofilm formation. No statistically significant difference was observed between fermented milk with 6% and 0.1% sucrose in the percentage of surface hardness loss.

Keywords: biofilm, enamel demineralization, fermented milk, *Lactobacillus paracasei*, probiotics, *Streptococcus mutans*

How to cite: Phimonsri R, Mitrakul K, Srisatjaluk R, **Asvanund Y**. Effect of low-sugar fermented milk with *Lactobacillus paracasei* 431 on *Streptococcus mutans* biofilm formation and enamel demineralization: in vitro study. M Dent J 2025;45(3): 248-265.

Introduction

Probiotics are live microorganisms that, when administered in sufficient quantities, provide health benefits to the host [1]. They work by interacting with intestine or oral bacteria, inhibiting harmful species through hydrogen peroxide, bacteriocins, and organic acids, and competing for nutrients and binding sites. Probiotics also modulate immune responses and metabolic processes [2]. Recent research on the oralome and its dysbiosis theory highlights the complex interactions between oral microorganisms and the host, impacting both microbial balance and health [3]. Probiotics show promise in maintaining or restoring healthy oral flora, potentially preventing or treating oral diseases. Several studies have investigated probiotic strains for dental caries prevention [4].

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Received: 31 July 2025 **Revised:** 21 November 2025 **Accepted:** 23 November 2025

Lactobacillus is commonly used in fermented dairy products and food supplements as a probiotic to support host health. Previous studies have shown that *Lactobacillus* strains help reduce the oral count of *Streptococcus mutans* and decrease caries incidence [2,4]. Various *Lactobacillus* strains, such as *L. acidophilus* LA-5, *L. casei* Shirota, *L. casei* LC01, *L. plantarum* ST-III, and *L. paracasei* LPC37, have demonstrated strong inhibitory effects on *S. mutans* growth [5]. In vitro studies also found that *L. paracasei* (11.6, 25.4,20.3) and *L. fermentum* could reduce *S. mutans* biofilm formation [6]. Fermented milk is a popular probiotic delivery system, but it often contains high levels of sucrose, which can increase the risk of dental caries.

Sucrose plays a key role in the cariogenicity of *S. mutans*. It serves as both a fermentable carbohydrate that drives acid production and a unique substrate required for extracellular polysaccharide (EPS) synthesis, which enhances bacterial adhesion and strengthens the biofilm matrix. High sucrose levels increase *S. mutans* acidogenicity and biofilm formation, promoting enamel demineralization [7]. Therefore, the sugar concentration in fermented products may influence *S. mutans* virulence and potentially counteract or modify the probiotic effects within the oral biofilm environment. Our previous studies showed that adding sucrose to plant-based milk enhanced *S. mutans* biofilm formation and enamel demineralization in primary teeth [8,9]. While Yakult® containing *L. casei* strain Shirota can suppress *S. mutans* growth despite its sugar content, its effectiveness diminishes when Lactobacilli are removed by filtering or heating [10]. In contrast, other studies report no significant change in salivary *S. mutans* levels after consuming Yakult® [11,12]. As consumer demand for healthier products grows, many fermented milk products are being modified to contain sugar substitutes. Currently, Dutch Mill Delight Imulus is the lowest sugar of single-species fermented milk available in Thailand. It contains *L. paracasei* 431 (IMULUS™) and only 0.1% sucrose because steviol glycoside and sucralose are used as sugar substitutes. A study found that consuming this product for 10 days significantly reduced salivary *S. mutans* levels ($p<0.001$) [13].

Dental caries is initiated in dental biofilm grown on the tooth surfaces [14]. Continuous consumption of high sucrose-containing diet can cause an ecological shift in the plaque microbiota, promoting acidogenic species and leading to tooth mineral loss [15]. Most fermented milk beverages are acidic and contain sugar, which can enhance the cariogenic potential of *S. mutans*. The low pH of these beverages can be associated with the development of dental caries and dental erosions [16]. However, an in vitro study showed that the mineral loss in enamel varied depending on the brand of fermented milk [17]. Another study found that probiotic fermented sheep milk with *Lactobacillus casei* 431 was effective in reducing enamel microhardness loss and surface changes [18]. However, this study used a pH cycling method without other microorganisms, which may not fully reflect the real effects of probiotics in the oral cavity.

Research on the cariogenic potential of reduced-sugar fermented milk products containing probiotics remains limited. In particular, the influence of *Lactobacillus paracasei* 431 on the growth and biofilm formation of *Streptococcus mutans* has not been clearly defined. Therefore, this study aims to investigate the effects of *Lactobacillus paracasei* 431 and sugar content in fermented milk on the growth and biofilm formation of *Streptococcus mutans*, as well as its potential to cause enamel demineralization. To confirm that the observed effects are attributable to *Lactobacillus paracasei* 431, the standard formulation will be compared with fermented milk without Lactobacilli.

Materials and Methods

This study method was approved by the Ethics Approval Review Board, Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University (COE.No.MU-DT/PY-IRB 2023/021. 2605).

Two formulas of fermented milk used in this study are shown in Table 1. Both contain *L. paracasei* 431 (IMULUS™). To investigate the effects of fermented milk without Lactobacilli, each fermented milk was filtered with a 0.22-micron filter (GVS Filtration Co., Italy) (Figure 1(a)). The filtered fermented milk was gram-stained and plated on BHI agar to confirm the absence of Lactobacilli.

Sample Size Calculation

For enamel surface hardness testing, the sample size was based on the mean ± SD of percentage of surface hardness loss of the fermented milk groups reported by Nadelman *et al.*, 2019 [19]. A priori power analysis using G*Power (v3.1.2; Universität Kiel, Germany) for one-way ANOVA (α = 0.05, power = 0.90). The number of calculated samples was at least eight slabs per group. In the present study, the sample size consisted of ten slabs per group, allowing for greater precision, accuracy, and a higher confidence level.

Bacteria preparation

S. mutans (ATCC 25175) was grown on BHI agar plate (Becton, Dickinson and Company, Franklin Lakes, NJ) at 37°C for 48 hours. Few colonies of *S. mutans* were transferred to fresh BHI broth and incubated under the same conditions until the mid-exponential phase (OD 600 nm = 0.5). *S. mutans* culture was then diluted to approximately 10^5 CFU/ml.

Table 1 Compositions of fermented milk used in this study

Name	Caloric density (kcal/mL)	Composition (g per 100 ml)					pH	*Lactobacillus* Strains
		Total CHO	Total Proteins	Total Fat	Total calcium	Sugars		
Fermented milk with 6% sucrose represented by the blue bottle (Dutch Mill Delight Imulus®)	0.50	11 g	2 g	0 g	0.048 g	Sucrose 6 g Lactose 3 g + Sucralose	4.02	*L. paracasei* 431 (IMULUS™)
Fermented milk with 0.1% sucrose represented by the green bottle (Dutch Mill Delight Imulus®)	0.19	4 g	1 g	0 g	0.048 g	Sucrose 0.1 g Lactose 2.9 g + Sucralose + Steviol glycoside	4.11	*L. paracasei* 431 (IMULUS™)

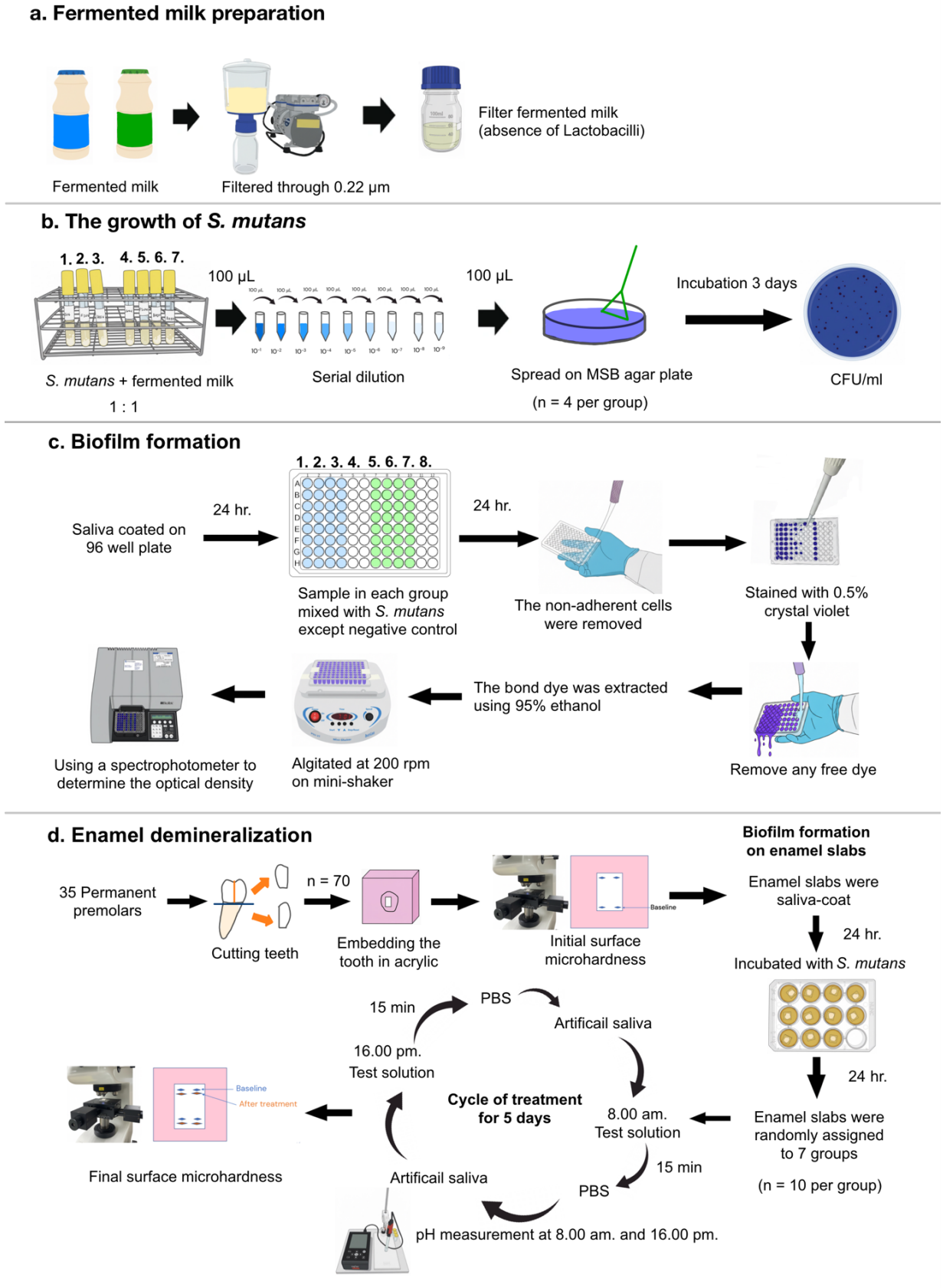

Figure 1 Schematic diagram of process steps

Effect of fermented milk on *S. mutans* growth

The quantity of *S. mutans* and *L. paracasei* was determined by the viable count. For baseline viable *S. mutans,* the culture was serially diluted, and 100 µL of each dilution was spread on MSB agar plates (Becton, Dickinson and Company, Franklin Lakes, NJ), incubated at 37°C for 72 hours. To determine the amount of *Lactobacillus* in the fermented milk, the fermented milk was serially diluted and 100 µL of each dilution was spread on Rogosa agar plates (Becton, Dickinson and Company, Franklin Lakes, NJ), incubated at 37°C for 72 hr. The bacterial colonies were recorded as colony-forming units CFU/ml.

To determine the effect of fermented milk on the growth of *S. mutans, S. mutans* suspension containing approximately 10^5 CFU/ml was mixed with various fermented milk samples in a 1:1 ratio. The fermented milk samples were 1) fermented milk with 6% sucrose (FM-6S), 2) filtered fermented milk with 6% sucrose (FFM-6S), 3) fermented milk with 0.1% sucrose (FM-0.1S), 4) filtered fermented milk with 0.1% sucrose (FFM-0.1S). *S. mutans* growth in the BHI without sucrose, the BHI with 6% sucrose, and the BHI with 0.1% sucrose served as positive controls. The mixed solution was incubated at 37°C for 15 hours. After incubation, the viable *S. mutans* and *L. paracasei* were determined as previously described (Figure 1(b)).

Saliva preparation

Stimulated saliva samples were obtained from three volunteers who refrained from tooth brushing for at least six hours prior to collection. The samples were pooled, centrifuged, diluted 1:10 with phosphate-buffered saline (PBS), and filtered through a 0.22-micron membrane. The prepared saliva was then used to coat 96-well polystyrene plates to promote *S. mutans* biofilm formation.

Artificial saliva was prepared to maintain moisture on the enamel slabs during the demineralization phase. Its composition included potassium chloride (0.65 g/L), magnesium chloride (0.058 g/L), calcium chloride (0.165 g/L), dipotassium hydrogen phosphate (0.804 g/L), potassium dihydrogen phosphate (0.365 g/L), sodium carboxymethyl cellulose (2 g/L), and deionized water (1 liter). The solution was filtered through a 0.22-micron membrane and stored at 4 °C until use [8].

Effect of fermented milk on *S. mutans* biofilm formation

S. mutans biofilm was formed on the 96-well plate and quantitated using the crystal violet assay. The sterile pooled human saliva (200 µL) was added to a 96-well plate (Corning Inc., Corning, NY) and incubated at 37°C for 24 hours. After saliva was removed, *S. mutans* (10^5 CFU/mL) was mixed with four groups of fermented milk samples in 1:1 ratio (100 µL each). The positive controls were *S. mutans* incubated with the BHI with 6% sucrose and the BHI with 0.1% sucrose. The negative controls were BHI with 6% and 0.1% sucrose. The 96-well plate was then incubated at 37°C for 24 hours. After incubation, non-adherent cells were removed by rinsing three times with tap water. The adherent biofilm cells were stained with 0.5% crystal violet (200 µL) for 15 minutes, followed by washing three times with tap water. After air-dried, 200 µL of 95% ethanol was added into each well to extract the crystal violet from the biofilm. The 96-well plate was agitated at 200 rpm for 15 minutes on a mini-shaker. The absorbance (OD) of the extracted solution was determined using a spectrophotometer (ELX800, Biotek, USA) at OD 590 nm [8] (Figure 1(c)).

Effect of fermented milk on enamel demineralization

To investigate the effect of fermented milk on enamel demineralization, the surface hardness of tooth specimens was measured. Specimens were obtained from 35 caries-free human permanent premolars, which were cut at the cementoenamel junction and split into two equal pieces of the buccal and lingual sides. Each tooth was mounted in a resin block, and its labial surface was polished with 100 to 5000-grit sandpaper and alumina powder until glossy. Initial surface hardness was assessed using Knoop indentations (FM-ARS 9000 Future-Tech Corp., Kanagawa, Japan), with hardness measured at four points using 50 grams of force for 15 seconds. The average initial surface hardness was 302.84 ± 5.43 kg/mm². Enamel slabs were sterilized before the experiment.

Enamel slabs were coated with pooled human saliva and incubated at 37°C for 24 hours. After removing the saliva, 3 ml of overnight-grown *S. mutans* in BHI broth with 0.5% sucrose (adjusted to 10^5 CFU/ml) was added to each well, followed by 24 hours of incubation to form biofilms. Seventy enamel slabs were randomly assigned to seven groups using a computer-generated randomization sequence (n = 10): 1) FM-6S, 2) FFM-6S, 3) FM-0.1S, 4) FFM-0.1S, 5) Positive control (BHI with 6% sucrose), 6) Positive control (BHI with 0.1% sucrose), and 7) Negative control (artificial saliva). There were no statistically significant differences in baseline hardness values across the groups ($p>0.05$).

Enamel slabs with *S. mutans* biofilms were immersed in 3 mL of each test solution for 15 min, twice daily at 8 a.m. and 4 p.m. to mimic a typical milk consumption pattern in children. After each exposure, the slabs were rinsed with phosphate-buffered saline (PBS) and returned to their respective wells containing artificial saliva, which were incubated at 37°C. This protocol was repeated for five consecutive days. After the experiment, surface hardness measurements were re-measured on specimens identified only by codes, with testing conducted randomly and without regard to group allocation, and the percentage of surface hardness loss (%SHL) was calculated as shown: (Mean initial SH – Mean final SH) x 100/ Initial SH

Biofilm acid production was assessed by measuring the culture medium pH twice daily before the replacement of the test solution (8:00 a.m. and 4:00 p.m.) using a pH meter (HM-40X, TOA DKK, Japan) with a glass combination electrode. Calibration was performed before each session using standard buffers (pH 4.01, 7.00, and 10.01). Measurements were conducted in triplicate with electrode rinsing between readings (Figure 1(d)).

Fluorescence staining and Confocal Laser Scanning Microscope (CLSM)

To evaluate cell viability and biofilm architecture on enamel, one slab per group was randomly selected after 5 days for CLSM analysis. Slabs were briefly rinsed with PBS (pH 7.0) to remove non-adherent cells and stained with the LIVE/DEAD® BacLight™ Kit L7012 (Invitrogen™, Molecular Probes Inc., OR, USA) for 15 minutes. Confocal images were obtained using a confocal laser scanning microscope (Stellaris8, Leica Microsystems, Germany) with a 63× oil immersion objective. Excitation lasers (488 nm for SYTO 9, 561 nm for propidium iodide) were set at 2% intensity. Z-stack images were captured at 1 µm intervals from the enamel surface to a height sufficient to capture the entire vertical structure of the biofilm. Image processing was performed using Leica LAS X software (Leica Microsystems, Wetzlar, Germany), including background subtraction and 3D reconstruction. Live (green) and dead (red) cell thresholds were initially auto-set. Subsequently, the lower intensity cut-off values for SYTO 9 and propidium iodide channels were manually adjusted, and these threshold values were then fixed and applied identically across all stacks to ensure consistency. The percentage of viable cells was calculated by quantifying the total bacterial pixel area (green + red) and the dead bacterial pixel area

(red) and expressed as: %Live = (total bacterial pixels – red pixels) / total bacterial pixels × 100 [20].

Statistical analyses

The normality of the data (CFU/mL, biofilm formation, and percentage of surface hardness loss) was assessed using the Shapiro–Wilk test. As the data did not follow a normally distributed, the Kruskal–Wallis test was employed to compare differences among groups, Bonferroni correction was applied to adjust *p*-values for multiple comparisons. The comparison scheme is summarized in Table 2. All statistical analyses were performed using SPSS version 28.0 (IBM Corp., Armonk, NY, USA), with a significance level set at $p<0.05$.

Results

Effect of fermented milk on *S. mutans* growth

The manufacturer claims that Dutch Mill Delight Imulus® contains at least 10^7 CFU/ml of *Lactobacillus*. To verify this, viable cell counts were conducted. The results indicated that FM-6S had an average viable bacterial concentration of $5.4x10^7$ CFU/ml (n=4), while FM-0.1S had an average concentration of $6.1x10^7$ CFU/ml (n=4).

The baseline concentration of *S. mutans* was $1.87×10^5$ CFU/ml (n = 4).

The growth of S. mutans incubated with different groups of fermented milk was measured. When comparing fermented milk with and without probiotics, the viable count of *S. mutans* in FM-6S ($1.51 \pm 0.15× 10^5$) was significantly lower than in FFM-6S ($1.11 \pm 0.25 × 10^7$) ($p<0.05$). Although FM-0.1S ($1.76 \pm 0.15x10^5$) also showed a lower viable count compared with FFM-0.1S ($0.87 \pm 0.10x10^7$), the difference was not statistically significant ($p>0.05$). When comparing fermented milk with different sucrose concentrations, no significant difference was observed between FM-0.1S ($1.76 \pm 0.15x10^5$) and FM-6S ($1.51 \pm 0.15x10^5$) ($p>0.05$). Likewise, the viable counts of FFM-0.1S ($0.87 \pm 0.10x10^7$) and FFM-6S ($1.11 \pm 0.25x10^7$) were not significantly different ($p>0.05$) (Figure 2(a)).

Table 2 Pairwise Comparison Scheme to Assess the Independent Effects of *Lactobacillus paracasei* 431 and Sugar Content.

Factor	Comparison groups	Rationale
Effect of *Lactobacillus paracasei* 431	FM-6S vs. FFM-6S FM-0.1S vs. FFM-0.1S	To isolate the effect of probiotics on *S. mutans* growth, biofilm formation, and enamel demineralization.
Effect of sugar content	FM-6S vs. FM-0.1S FFM-6S vs. FFM-0.1S	To isolate the effect of sugar content on *S. mutans* growth, biofilm formation, and enamel demineralization.
Comparison to positive control	FM-6S vs. Pos BHI-6S FFM-6S vs. Pos BHI-6S FM-0.1S vs. Pos BHI-0.1S FFM-0.1S vs. Pos BHI-0.1S	To interpret the cariogenic potential of the fermented milk relative to a high-cariogenic reference condition.

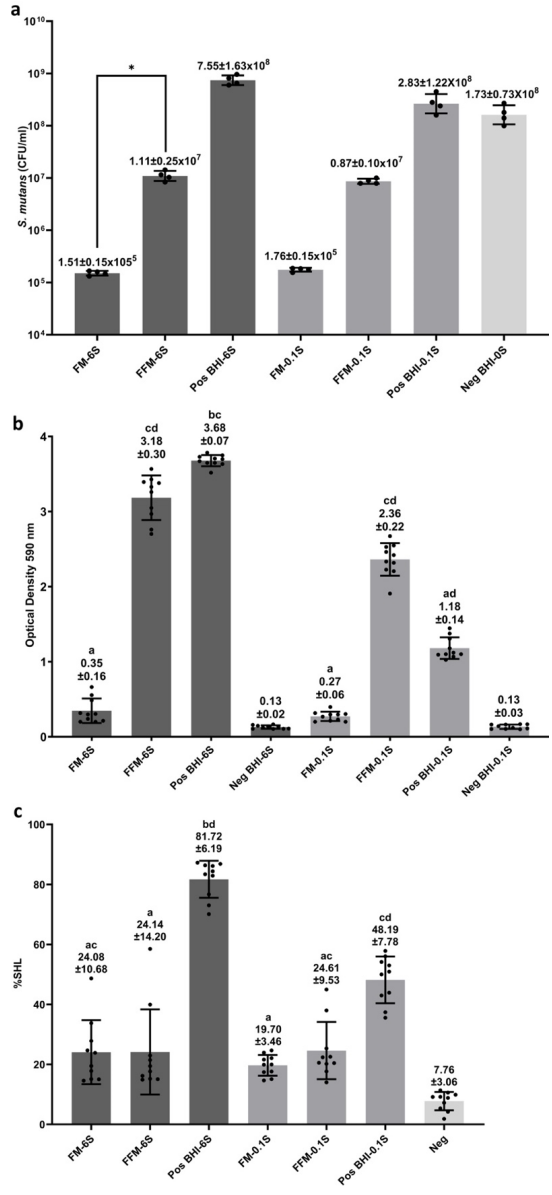

Figure 2 (a) The growth of *S. mutans* after incubation with various tested solutions (n=4). Data are presented as mean CFU/mL ± SD (error bars), dots representing individual values (n). *p<0.05 indicates statistical significance based on the Kruskal–Wallis test with Bonferroni correction. (b) Optical density at 590 nm after staining biofilms with 0.5% crystal violet (n = 10). Data are presented as means ± SD, with dots representing individual values (n). Different superscript letters indicate significant differences (p<0.05) according to the Kruskal–Wallis test with Bonferroni correction. (c) The percentage of surface hardness loss (%SHL) after experiment (n = 10). Data are presented as means ± SD, with dots representing individual values (n). Different superscript letters indicate significant differences (p<0.05) according to the Kruskal–Wallis test with Bonferroni correction. (FM-6S = Fermented milk with 6% sucrose, FFM-6S = Filter fermented milk with 6% sucrose, Pos BHI-6S = Positive control (BHI with 6% sucrose), FM-0.1S = Fermented milk with 0.1% sucrose, FFM-0.1S = Filter fermented milk with 0.1% sucrose, Pos BHI-0.1S = Positive control (BHI with 0.1% sucrose), Neg BHI-0S = Negative control (BHI without sucrose), Neg BHI-6S = Negative control (BHI with 6% sucrose), Neg BHI-0.1S = Negative control (BHI with 0.1% sucrose))

Effect of the fermented milk on *S. mutans* biofilm formation

After 24 hours of incubation, FM-6S (0.35 ± 0.16), which contained probiotics, exhibited significantly reduced biofilm formation compared with FFM-6S (3.18 ± 0.30) (p<0.001). Similarly, FM-0.1S (0.27 ± 0.06) showed significantly lower biofilm formation than FFM-0.1S (2.36 ± 0.22) (p<0.05). When comparing sucrose concentrations within the fermented milk groups, FM-0.1S (0.27 ± 0.06) showed a lower biofilm formation than FM-6S (0.35 ± 0.16), although the difference was not statistically significant (p>0.05). A similar trend was observed in the filtered fermented milk groups, where FFM-0.1S (2.36 ± 0.22) exhibited lower biofilm formation than FFM-6S (3.18 ± 0.30), but without statistical significance (p>0.05). Fermented milk also reduced biofilm formation compared with the positive control. FM-6S (0.35 ± 0.16) was significantly lower than Pos BHI-6S (3.68 ± 0.07) (p<0.05), while FM-0.1S (0.27 ± 0.06) was lower than Pos BHI-0.1S (1.18 ± 0.14), but not significantly (p>0.05). In contrast, the filtered samples, FFM-6S (3.18 ± 0.30) and FFM-0.1S (2.36 ± 0.22), showed no significant differences compared with Pos BHI-6S (3.68 ± 0.07) and Pos BHI-0.1S (1.18 ± 0.14), respectively (Figure 2(b)).

Effect of fermented milk on enamel demineralization

After the 5-day treatment, all fermented milk groups demonstrated reduced enamel surface hardness loss (SHL) (Table 3, Figure 2(c)). In the comparison between products with and without probiotics, FM-6S (24.08 ± 10.68) exhibited an SHL value comparable to FFM-6S (24.14 ± 14.20). Although FM-0.1S (19.70 ± 3.46) showed a lower SHL than FFM-0.1S (24.61 ± 9.53), the difference was not statistically significant (p>0.05). Regarding sugar concentrations, FM-0.1S (19.70 ± 3.46) showed less demineralization than FM-6S (24.08 ± 10.68), but the difference was not statistically significant (p>0.05). Likewise, FFM-6S (24.61 ± 9.53) demonstrated a comparable SHL to FFM-0.1S (24.14 ± 14.20), with no statistically significant difference (p>0.05). Fermented milk groups exhibited significantly lower SHL than the respective positive controls (p<0.05), including FM-6S (24.08 ± 10.68) and FFM-6S (24.14 ± 14.20) versus Pos BHI-6S (81.72 ± 6.19), and FM-0.1S (19.70 ± 3.46) versus Pos BHI-0.1S (48.19 ± 7.78). These findings indicate that fermented milk markedly attenuated enamel demineralization compared with the positive controls.

Table 3 Surface microhardness value (mean ± SD) at baseline, after experiment, and the percentage of surface hardness loss (%SHL)

Experiment and control groups	Baseline (Mean±SD)	After experiment (Mean±SD)	%SHL (Mean±SD)
Fermented milk with 6% sucrose	301.80 ± 7.82	233.86 ± 21.99	24.08 ± 10.68^{ac}
Filter fermented milk with 6% sucrose	303.32 ± 8.03	229.97 ± 42.13	24.14 ± 14.20^{a}
Positive control (BHI with 6% sucrose)	304.20 ± 3.19	55.29 ± 18.26	81.72 ± 6.19^{bd}
Fermented milk with 0.1% sucrose	303.83 ± 2.44	244.02 ± 11.46	19.70 ± 3.46^{a}
Filter fermented milk with 0.1% sucrose	304.09 ± 2.36	228.74 ± 28.44	24.61 ± 9.53^{ac}
Positive control (BHI with 0.1% sucrose)	303.18 ± 2.67	166.42 ± 35.79	48.19 ± 7.78^{cd}
Negative control (artificial saliva)	301.33 ± 7.60	277.89 ± 10.63	7.76 ± 3.06

Different superscript letters indicate significant differences (p<0.05) based on the Kruskal-Wallis test with Bonferroni correction

The pH values during a 5-day enamel demineralization experiment are presented in Figure 3. The pH ranged from 6.22 to 6.79 in solutions containing fermented milk and filtered fermented milk with 6% or 0.1% sucrose, with no significant difference between them. However, on day 2, the pH of the positive control (BHI 6% sucrose) dropped to 4.5.

Effect of fermented milk on cell viability and architecture of *S. mutans* biofilm

Three-dimensional images of biofilms are presented in Figures 4–6. In fermented milk, the biofilms appeared looser than those observed in the positive control at equal sucrose concentrations. Specifically, the positive control (BHI with 6% sucrose) displayed a dense, mushroom-like architecture (Figure 4). *L. paracasei* rods were clearly observed on enamel surfaces in fermented milk, but they were absent in filtered fermented milk (Figure 5). Moreover, biofilms formed in fermented milk were thinner compared to those in the positive control under the same sucrose concentrations. Within the fermented milk groups, FM-6S biofilms were noticeably thicker than FM-0.1S (Figure 6 (a, b)). Importantly, FM-0.1S biofilms contained a higher proportion of dead cells than FM-6S, while both fermented milk groups with Lactobacilli exhibited more dead cells than the filtered fermented milk group (Figure 6 (c)).

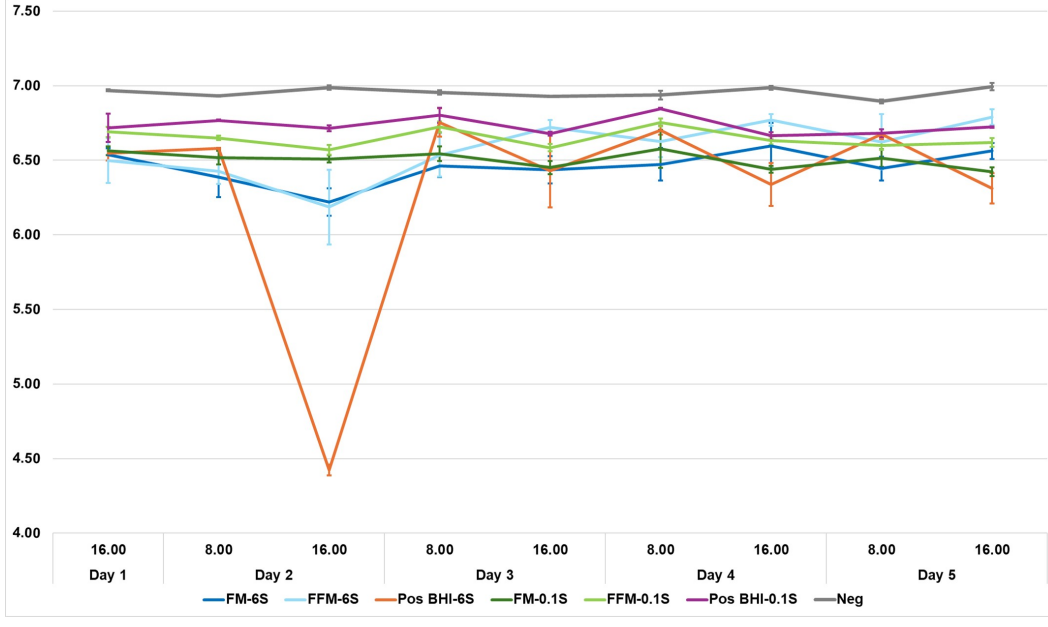

Figure 3 pH values of the solution during the 5-day enamel demineralization experiment. Data are presented as means ± SD (error bars), with three samples per group (n = 3) and three technical replicates measured for each sample. (FM-6S = Fermented milk with 6% sucrose, FFM-6S = Filter fermented milk with 6% sucrose, Pos BHI-6S = Positive control (BHI 6% sucrose), FM-0.1S = Fermented milk with 0.1% sucrose, FFM-0.1S = Filter fermented milk with 0.1% sucrose, Pos BHI-0.1S = Positive control (BHI 0.1% sucrose), Neg = Negative control (artificial saliva))

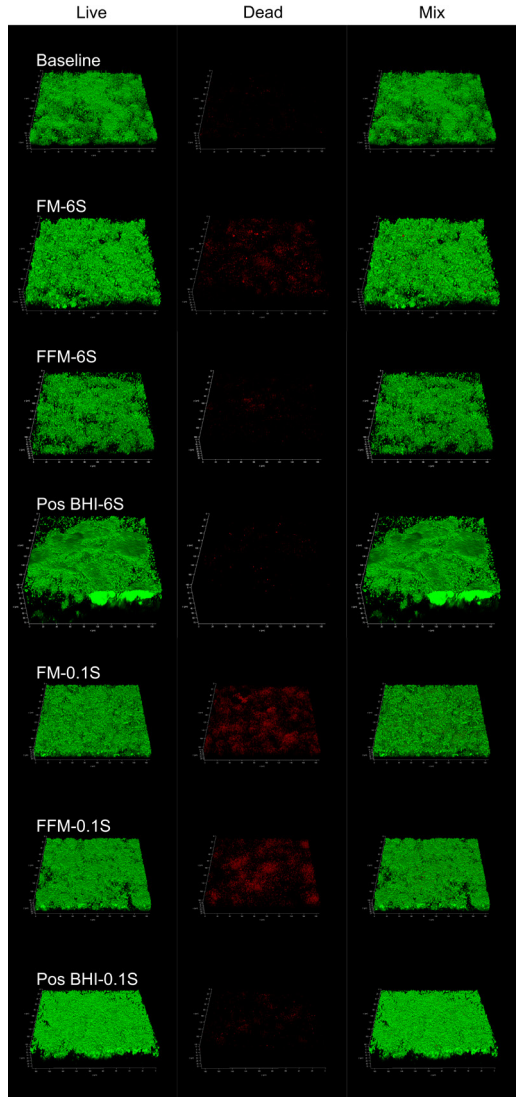

Figure 4 Three-dimensional architecture of biofilms on permanent teeth enamel slab visualized by confocal laser scanning microscope. Live cells were stained green and dead cells were stained red. (FM-6S = Fermented milk with 6% sucrose, FFM-6S = Filter fermented milk with 6% sucrose, Pos BHI-6S = Positive control (BHI with 6% sucrose), FM-0.1S = Fermented milk with 0.1% sucrose, FFM-0.1S = Filter fermented milk with 0.1% sucrose, Pos BHI-0.1S = Positive control (BHI with 0.1% sucrose))

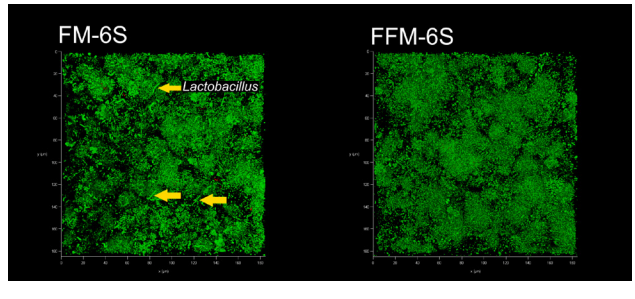

Figure 5 The rod-shaped of *L. paracasei* were observed in the specimens treated with the fermented milk. (FM-6S = Fermented milk with 6% sucrose, FFM-6S = Filter fermented milk with 6% sucrose)

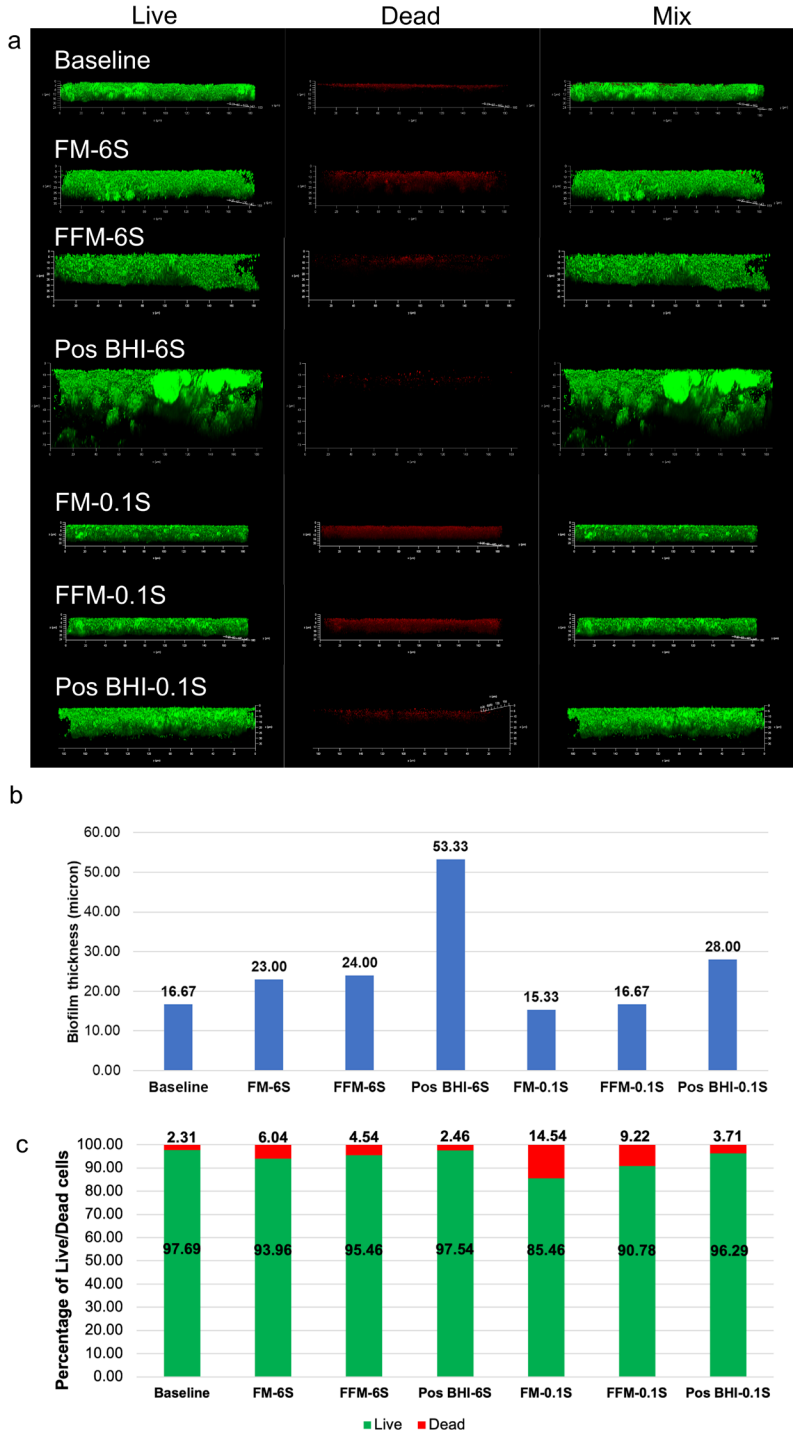

Figure 6 (a) The thickness of biofilms on permanent teeth enamel slab visualized by CLSM. (b) Quantitative measurements of biofilm thickness (μm) of biofilms on permanent teeth enamel slab visualized by CLSM. (c) Percentage of live and dead cells of S. *mutans* in biofilms on enamel slab using a confocal laser scanning microscope. Green indicates live cells, and red indicates dead cells. (FM-6S = Fermented milk with 6% sucrose, FFM-6S = Filter fermented milk with 6% sucrose, Pos BHI-6S = Positive control (BHI with 6% sucrose), FM-0.1S = Fermented milk with 0.1% sucrose, FFM-0.1S = Filter fermented milk with 0.1% sucrose, Pos BHI-0.1S = Positive control (BHI with 0.1% sucrose)

Discussion

Fermented milk is widely utilized as a probiotic delivery system. However, its cariogenic potential remains unclear. Although probiotics have been associated with caries-preventive effects, the low pH and fermentable sugars in fermented milk may still promote enamel demineralization. Accordingly, this study aims to investigate the effects of *Lactobacillus paracasei* 431 and sugar content in fermented milk on the growth and biofilm formation of *Streptococcus mutans*, as well as its potential to cause enamel demineralization.

In this study, a dual-species model of *S. mutans* and *L. paracasei* 431 was used to evaluate the direct inhibitory effect of the probiotic on *S. mutans* growth and biofilm formation. Our results show that fermented milk containing viable *L. paracasei* 431 significantly reduced *S. mutans* counts compared with filtered fermented milk.

Since filtration removes the probiotic cells while retaining soluble fermentation-derived metabolites such as lactic acid and bacteriocins, the diminished inhibitory activity observed in the filtered samples indicates that viable *L. paracasei* rather than metabolites alone are essential to suppression of *S. mutans*. These findings align with previous research showing that Yakult®, which contains the *L. casei* strain Shirota, also inhibits *S. mutans* growth, though its effect is weakened after filtration or heating [10]. Consistently, biofilm formation of *S. mutans* was significantly lower in the presence of fermented milk than in both positive controls and the filtered fermented milk. Although reduced nutrient availability in fermented milk compared with BHI may partially contribute to this decrease, it does not fully account for the observed effect. When *S. mutans* in BHI was mixed 1:1 with fermented milk, the samples containing viable *Lactobacillus* still exhibited significantly lower biofilm formation than the filtered fermented milk. These findings support the conclusion that the inhibitory effect arises not only from media composition but from an intrinsic anti-biofilm activity of *Lactobacillus*, consistent with previous reports [21–24].

Several mechanisms may explain the inhibitory effects observed in this study. First, *Lactobacillus* species can produce antimicrobial substances such as bacteriocins, organic acids, and hydrogen peroxide, which inhibit the growth of *S. mutans* [21]. Second, these probiotics interfere with bacterial adhesion to oral surfaces. *In vitro* studies have demonstrated that *L. reuteri* PTA 5289 and *L. paracasei* DSMZ 16671 significantly reduce the adhesion of *S. mutans* to hydroxyapatite [22]. Similarly, *L. paracasei* ET-22 inhibited initial *S. mutans* adhesion by altering the interactions between the bacteria and the tooth surface [23]. Third, *Lactobacillus* species have been found to downregulate genes associated with biofilm formation. For example, *L. paracasei* 28.4, when incorporated into gellan-based hydrogels, inhibited biofilm formation by suppressing the expression of critical biofilm-related genes (*luxS*, *brpA*, *gbpB*, *gtfB*) and reducing extracellular polysaccharide synthesis [24]. In addition to laboratory findings, several clinical studies have reported the effects of *Lactobacillus* on *S. mutans*. For example, milk supplemented with *Lactobacillus paracasei* SD1 was shown to reduce salivary *S. mutans* and decrease the risk of dental caries in high-risk groups [25]. Together, these mechanisms support our findings that *L. paracasei* 431 exerts a multifactorial inhibitory effect on both the growth and biofilm formation of *S. mutans*.

In this study, fermented milk containing 0.1% sucrose showed lower *S. mutans* biofilm formation than the 6% sucrose formulation, although the difference did not reach statistical significance.

CLSM analysis similarly revealed thinner biofilms on enamel slabs in the 0.1% sucrose group (Figure 6 (b)). These findings suggest that reducing sucrose concentration may modestly attenuate biofilm development and cariogenic potential; however, the lack of significance indicates that sucrose reduction alone is insufficient due to the multifactorial nature of biofilm formation. The 0.1% sucrose fermented milk also contained steviol glycoside, a compound previously shown to suppress *S. mutans* biofilm formation, decrease EPS production, and downregulate EPS-related genes (*Gtfs*, *Gbps*) [26]. In addition, steviol glycoside has demonstrated antimicrobial properties, with MIC and MBIC values of 25 mg/mL and 6.25 mg/mL, respectively [27]. Nevertheless, the concentration of steviol glycoside in our formulation was not specified, which limits direct comparison with prior studies and may explain why its inhibitory effect was not clearly observed. Another critical factor is the presence of multiple sweeteners in the formulation, including sucrose, lactose, and sucralose in addition to steviol glycoside. Sucrose remains the most potent stimulator of *S. mutans* biofilm formation [7], while lactose and sucralose also promote biofilm accumulation, though to a lesser extent [28,29]. The coexistence of these sugars likely confounded the outcomes, as their combined effects may have masked the potential benefits of sucrose reduction and steviol glycoside supplementation.

Our results demonstrated the inhibitory effects of fermented milk on *S. mutans* growth and biofilm formation. We further determined the effects of fermented milk on enamel demineralization. The results showed a significantly lower surface hardness loss in the presence of either fermented milk or filtered fermented milk compared with the positive controls, as evidenced by FM-6S (24.08 ± 10.68) and FFM-6S (24.14 ± 14.20) versus Pos BHI-6S (81.72 ± 6.19), and FM-0.1S (19.70 ± 3.46) versus Pos BHI-0.1S (48.19 ± 7.78). Although the fermented milk used in this study had a pH value of approximately 4, which is lower than the critical pH for demineralizing dental enamel, which is 5.5 [30]. The pH values during the enamel demineralization experiment were around 6 to 7, possibly due to the buffering effect of artificial saliva. Fermented milk contains cariostatic function as an acid buffer, which is important for lowering demineralization [31]. Previous studies have found that probiotic drinks have low erosive activity due to their high calcium content and specific bacterial strains [32]. Casein, the primary protein in milk, may help sustain the high calcium phosphate content in milk, preventing precipitation [33]. Previous study found that kefir fermented milk, which contains casein phosphopeptides that bind with calcium phosphate, has a protective effect on enamel demineralization, although the pH was 4.5 [34]. Although a reduction in enamel surface hardness was observed after exposure to fermented milk containing 6% and 0.1% sucrose (233.86 ± 21.99 kg/nm² and 244.02 ± 11.46 kg/nm², respectively), the values remained comparable to those found in early enamel lesions (ICDAS code 1) [35], which are considered clinically reversible through remineralization therapies [36]. In contrast, the positive control groups with equivalent sucrose concentrations showed significantly lower mean surface hardness (55.29 ± 18.26 kg/nm² for 6% sucrose and 166.42 ± 35.79 kg/nm² for 0.1% sucrose), corresponding to ICDAS codes 3 and 2, respectively [35]. The present findings suggest that fermented milk results in less enamel demineralization compared to sucrose solution, with effects lower than anticipated. Therefore, drinking low-sugar fermented milk is an alternative consumption option for patients with health problems, such as improving the gastrointestinal tract or enhancing the immune system [37]. Nonetheless, further long-term studies are needed to confirm these results and determine the safety margin.

In this study, we compared the effects of fermented milk with and without *L. paracasei* 431 on enamel demineralization using *S. mutans* biofilm model. After 5 days of experiment, our samples showed no significant differences %SHL between probiotic and non-probiotic formulations. FM-6S (24.08 ± 10.68) and FFM-6S (24.14 ± 14.20) exhibited comparable %SHL values, while FM-0.1S (19.70 ± 3.46) was slightly lower than FFM-0.1S (24.61 ± 9.53), although the difference was not statistically significant. These findings indicate that the presence of *L. paracasei* 431 did not markedly influence enamel surface hardness loss under the conditions tested. Our findings align with previous research on fermented sheep milk containing *Lactobacillus casei* 431 using a pH-cycling model. In that study, conventional fermented milk prepared with *Lactococcus lactis* LR-35® (a non-probiotic starter) and probiotic fermented milk with *L. casei* 431 both reduced enamel demineralization, showing comparable %SHL values (35.18 ± 8.75 and 33.66 ± 14.14, $p>0.05$) [18]. Likewise, another study using mixed biofilms of *S. mutans* and *L. casei* 01 reported no prevention of surface hardness loss, although a trend toward reduced subsurface demineralization was observed relative to conventional fermented milk prepared with *L. lactis* [19]. However, previous study showed significantly greater mineral loss in *S. mutans* single-species biofilms compared with mixed biofilms containing *S. mutans* and *L. casei* LC-11, indicating that *L. casei* can reduce *S. mutans* biofilm formation and subsequently decrease enamel demineralization [38]. Our results and previous evidence indicate that, although *L. casei* may enhance the protective effect in a strain-dependent manner, the primary prevention of enamel demineralization is mainly attributed to the calcium, phosphate, and casein naturally present in fermented milk.

Prior to the experiment, we hypothesized that fermented milk containing 0.1% sucrose would result in lower enamel surface hardness loss (%SHL) than fermented milk containing 6% sucrose, based on evidence that sucrose supplementation increases the cariogenicity of milk [39]. However, FM-0.1S (19.70 ± 3.46) and FM-6S (24.08 ± 10.68) did not differ significantly ($p>0.05$), nor did FFM-0.1S (24.14 ± 14.20) and FFM-6S (24.61 ± 9.53) ($p>0.05$). These findings suggest that fermented milk may help attenuate enamel demineralization; however, the influence of sucrose concentration was not clearly demonstrated in this study.

A confocal laser scanning microscope (CLSM) was used to examine the effect of fermented milk on biofilm formation in three-dimensional images. Our study found that biofilms in fermented milk were thinner and less dense than those in the positive control group with the same sucrose concentrations. This is similar to a previous study where co-cultures of *S. mutans* and *Lactobacillus plantarum* K41 showed reduced biofilm density and thinner biofilms [40]. We also observed more dead cells in biofilms exposed to fermented milk with 0.1% sucrose compared to 6% sucrose. This could be due to steviol glycoside in the 0.1% sucrose formula, which can reduce biofilm viability and EPS production. However, sucrose might lessen the impact compared to steviol glycoside alone [41]. Additionally, biofilm characteristics were linked to enamel demineralization, as previous studies have shown that localized enamel demineralization is influenced by the EPS-rich matrix around bacterial microcolonies [42].

The present study used a dual-species biofilm model under static conditions. While this design was appropriate for testing direct interactions between *S. mutans* and *L. paracasei*, it does not fully represent the multispecies dynamics and environmental conditions of the oral cavity.

The absence of salivary flow excludes shear forces and nutrient clearance in the oral cavity, and the short incubation time restricts evaluation of long-term enamel demineralization. Moreover, the presence of multiple sugars in the fermented milk may have acted as confounding factors. In addition, the lack of statistical significance between the FM-0.1S and FFM-0.1S groups in the CFU analysis may be attributed to limited statistical power due to the small sample size (n = 4). Future studies should incorporate dynamic flow conditions, extend the experimental duration, control the sugar composition, and increase the sample size in the CFU experiment to better mimic in vivo conditions and enhance clinical relevance.

Conclusion

Fermented milk with *Lactobacillus paracasei* 431 inhibited *S. mutans* growth and biofilm formation. However, its effect on enamel demineralization was comparable to the non-probiotic formulation in the 6% sucrose groups and showed only a non-significant reduction in the 0.1% sucrose groups. When comparing sugar concentrations, the 0.1% sucrose formulations demonstrated lower biofilm formation and surface hardness loss than the 6% sucrose formulations, although these differences were not statistically significant. These findings suggest that while the probiotic and reduced sucrose levels can suppress bacterial activity, their protective effect on enamel under the tested conditions remains limited.

Acknowledgements

The authors thank Mr. Arthit Klaophimai and the senior staff of the Research Service Center of the Faculty of Dentistry, Mahidol University.

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