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Original Article

Effects of Alternative Milk on *Streptococcus Mutans* Biofilm Formation and Enamel Demineralization in Human Primary Teeth

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Abstract

Objectives This study was conducted to investigate the effects of alternative milk on *Streptococcus mutans* biofilm formation and their ability to demineralize enamel in primary teeth.

Materials and Methods First, to evaluate the effects of cow milk, lactose-free cow milk, goat milk, unsweetened pistachio milk, and sweetened pistachio milk on *S. mutans* biofilm formation, biofilm assay was conducted. The optical density (OD) was measured to determine *S. mutans* biofilm. Second, to assess the enamel demineralization, enamel slabs were prepared from 50 primary incisor teeth and divided into three test groups, along with positive and negative control groups. Enamel slabs were immersed in each type of milk three times a day for 5 days. The percentage of surface hardness loss (%SHL) for enamel demineralization was measured. One enamel slab was randomly selected from each group to visualize the enamel opacity in demineralization area by using a light microscope. Another slab was randomly selected from each group to stain with fluorescence dye and to observe the biofilm structure by using a confocal microscope.

Results The OD ± SD (standard deviation) measurements for *S. mutans* biofilm formation in cow milk, lactose-free cow milk, goat milk, unsweetened pistachio milk, and sweetened pistachio milk were 0.082 (± 0.002), 0.086 (± 0.004), 0.083 (± 0.007), 0.0952 (± 0.010), and 0.342 (± 0.072), respectively. The sweetened pistachio milk exhibited significantly more biofilm formation than the other milk ($p < 0.05$). Since there was no significant difference in biofilm formation among cow milk, lactose-free cow milk, goat milk, and unsweetened pistachio milk, we tested the enamel demineralization only with cow milk, unsweetened pistachio milk, and sweetened pistachio milk. The %SHL (± SD) for cow milk, unsweetened pistachio milk, and sweetened pistachio milk were 20.01 (± 2.618), 22.088 (± 3.4), and 35.49 (± 2.069), respectively. The %SHL on the enamel in sweetened pistachio milk was higher ($p < 0.001$) than other tested milk. White spot lesion was directly visualized on slabs in sweetened pistachio milk under light microscope. Biofilm formed in sweetened

Keywords
- alternative milk
- biofilm
- enamel demineralization
- streptococcus mutans

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pistachio milk demonstrated a mushroom-like structure, whereas cow milk and unsweetened pistachio milk had a flat biofilm structure.

Conclusion Sweetened pistachio milk increases the risk for primary incisor teeth caries formation greater than cow milk and unsweetened pistachio milk regarding *S. mutans* biofilm formation was higher in quantity and ability to demineralization in primary teeth.

Introduction

Dental caries is a multifactorial disease. It belongs to the group of common diseases considered as "complex" or "multifactorial" such as cancer, heart diseases, diabetes, and certain psychiatric illnesses. There is no simple causation pathway.[1] In the oral cavity, there exists biofilm, or dental plaque, which comprised of more than 800 species of microorganisms living together.[2] Dental caries is provoked by the dysbiosis caused in biofilm due to frequent sugar intake.[2] To have dental caries, it is necessary to accumulate biofilm and expose it frequently to fermentable carbohydrates, which is fermented by multispecies of bacteria. The acids released into the aqueous part of the biofilm provoke the enamel/dentine demineralization. The low pH environment favors acidogenic and aciduric bacterial selection.[3,4]

Streptococcus mutans is a microorganism commonly isolated from dental plaque and strongly associated with dental caries.[5–9] The major virulence traits of *S. mutans* are its acidogenicity that exacerbates the damage to dental hard tissues; its aciduricity that contributes to its survival in low pH environments or to out-compete other oral bacteria; and its ability to synthesize insoluble exopolysaccharides (EPS) from sucrose, which is involved in the initial attachment, colonization, and accumulation of dental plaque.[10,11] Over time, more acid is produced as a consequence of sugar fermentation, lowering the pH of the dental biofilm to below 5.5, which is the critical pH threshold for enamel demineralization.[11] Studies in Thai children reported that the number of *S. mutans* in plaque is greater in children with early childhood caries than those who are caries free.[8,9]

Cow milk is one of the most consumed foods worldwide. It is a good source of carbohydrates, proteins, fats, vitamins, calcium, and phosphate.[12] Several studies have reported cow milk contains casein, fat, and various antimicrobial peptides, such as lactoferrin, lysozyme, and peroxide, which may contribute to anticariogenicity.[12–15] Also, lactose, a disaccharide sugar present in cow milk, is less cariogenic than sucrose.[13,15]

Goat milk is an alternative to cow milk because its protein is easier to digest because the fat globules are much smaller and easier on the digestive system. It has more calcium, and less lactose.[16] Goat milk represents 13.5% of nonbovine milk production worldwide, making it the most significant contributor. Other health benefits that have been identified include being easier to digest and less risk of milk allergies when compared with cow milk due to different kind of protein and potentially that are better for heart health.[17] Some studies have reported that it can be consumed by most of the population with lactose intolerance.[17]

In some populations, consuming cow milk is not possible. One reason for this is allergy to the protein in cow milk or lactose intolerance. Other reasons are ethical concerns about animal rights, the popularity of plant-based diets and the perception that plant-based milk is healthier than cow milk.[15,18,19] This has led to the increased popularity of plant-based milk. Plant-based milk is the fluid resulting from the breakdown of plant materials, such as almond, soy, oat, pistachio, and other varieties of nuts. It is becoming increasingly popular due to the popularity of plant-based diets or vegetarianism. Several studies have compared the effect of plant-based milk and cow milk on *S. mutans* biofilm formation.[13,20] These studies found that plant-based milk produced less biofilm, but had a poorer buffering capacity compared with cow milk.[13] Plant-based milk may be sold as sweetened or unsweetened. However, sucrose is often added to plant-based milk to improve the taste to help it sell better. When sugar was added to plant-based milk, it produced thicker biofilm compared with cow milk.[13,15] Another study investigating milk's effect on demineralized enamel surfaces on permanent teeth found that cow milk and plant-based milk demonstrated no difference.[15] A previous study found that soy milk supported a significantly higher yield of *S. mutans* and higher quantities of acid production from fermentation. Furthermore, it also had a lower buffering capacity compared with cow milk, which suggests that soy milk has a higher cariogenic potential than cow milk.[15] Our previous study compared the demineralization ability of cow milk, sweetened almond milk, and sweetened soy milk on the enamel surfaces in primary teeth and found that sweetened almond milk had a significantly lower percentage of surface hardness loss (%SHL) than sweetened soy milk and bovine milk.[20] There are numerous plant-based milk products commercially available in the market; however, little data are available on the effect of these products on their risk for contributing to caries formation, especially in primary teeth.

The aim of this study was to determine *S. mutans* biofilm formation and enamel demineralization caused by cow milk, lactose-free cow milk, goat milk, and sweetened and unsweetened pistachio milk and to evaluate their risk for contributing to caries formation in human primary incisor teeth.

Materials and Methods

Bacteria Preparation

S. mutans standard strain (ATCC 25175) was grown on a brain heart infusion (BHI) agar plate (Becton, Dickinson and Company, Franklin Lakes, New Jersey, United States) in 5% carbon dioxide (CO_2) at 37°C for 48 hours. A sample from the plate was inoculated into fresh BHI broth and grown under the same conditions until it reached the midexponential phase (optical density $[OD]_{600\,nm} = 0.5$). The *S. mutans* culture was then diluted to 10^6 colony-forming units (CFU) per milliliter.

Saliva Preparation

Stimulated pooled saliva was obtained from three volunteers who abstained from tooth brushing for at least 6 hours prior to saliva collection. The saliva samples were pooled, centrifuged, and then diluted to 1:10 with phosphate-buffered saline (PBS) and filtered through an autoclaved 0.22-µm membrane. The objectives to use saliva were to coat the wells of a 96-well and 24-well polystyrene plates to initiate *S. mutans* biofilm formation, since it adheres to the glycoprotein in the saliva and helps keep the enamel slabs moist during the enamel demineralization assay. Each well containing 500 µL of diluted saliva was incubated at 37°C for 24 hours and then the saliva rinsed off.

For the enamel demineralization part of the study to keep the enamel slabs moist during the artificial saliva was prepared and filtered through a 0.22-µm membrane and kept at 4°C until use.[15]

S. mutans Biofilm Amount Determination

Saliva-coated 96-well plate was prepared for biofilm formation by adding 200 µL sterile pooled human saliva to each well, incubating for 24 hours. The saliva was removed, 100 µL *S. mutans* (10^6 CFU/mL) prepared as described above, and was inoculated into each well. This was done in triplicate for each test milk. The five test milk products were cow milk, lactose-free cow milk, goat milk, unsweetened pistachio milk, and sweetened pistachio milk (►**Table 1**). Each test milk was diluted with sterile deionized water to a milk-to-water ratio of 1:2. BHI broth with 5% sucrose and sterile deionized water were used as positive and negative controls, respectively, and were added to their respective wells. The plate was gently agitated and incubated in 5% CO_2 at 37°C for 24 hours to allow biofilm formation. After incubation, the plate was agitated at 200 rpm for 10 minutes on a shaker (Mini-Shaker PSU-2T, Biosan, Rīga, Latvia) and each well was gently washed with running tap water to remove nonadherent cells. The remaining biofilm in each well was stained with 0.05% crystal violet solution for 10 minutes and the unbound dye was rinsed out with running tap water. The bound dye was extracted by adding a mixture of ethanol and acetone (4:1) for 10 minutes. An aliquot of this dye-containing solution was transferred to a new 96-well plate and the OD at 575 nm was measured using a micro-plate reader (Bio-Tek Instruments Inc., Winooski, Vermont, United States). The absorbance indicated the amount of biofilm present in each well.

Enamel Demineralization Assay

There was no significant difference in the *S. mutans* biofilm formation among the cow milk, lactose-free cow milk, goat milk, and unsweetened pistachio milk. Therefore, we performed the demienralization assay using only cow milk, unsweetened pistachio milk, and sweetened pistachio milk, as discussed in the Results section below.

Table 1 Alternative milk used in this study

Type		Composition (g per 100 mL)				
Name/manufactures	Abbreviation	Total CHO	Total proteins	Total fat	Sugars	Sugars added
Whole cow milk (Nongpho UHT, Nongpho Ratchaburi Dairy Cooperative Ltd., Thailand)	COW	4.4	3.1	3.5	4Lactose	–
Lactose-free cow milk (M Milk UHT, Mary Anne Dairy Product Cooperative Ltd., Thailand)	LAC	4.8	3.2	3.6	1.6Lactose	–
Goat milk (Sirichai UHT, Mary Anne Dairy Product Cooperative Ltd., Thailand)	GOA	4.2	3.2	2.6	3.7Lactose	–
Unsweetened pistachio milk (Sunkist, Heritage Snacks & Food Cooperative Ltd., Thailand)	PUN	3.9	< 0.5	1	3.9Sucrose +Glucose +Fructose +Maltose	–
Sweetened pistachio milk (Sunkist, Heritage Snacks & Food Cooperative Ltd., Thailand)	PSW	6.7	< 0.5	1	3.4Sucrose +Glucose +Fructose +Maltose	3.3 (Raw cane sugar: sucrose)

For the enamel demineralization assay, we obtained human primary incisor teeth that had normally exfoliated or been extracted because of prolonged retention from the Department of Pediatric Dentistry at Mahidol University and private clinics in Bangkok, Thailand. Fifty caries-free primary incisor teeth were obtained and stored in 0.1% thymol solution at room temperature. Each tooth was decoronated at the cementoenamel junction. An enamel window was made by covering the labial middle third of each tooth with a $4 \times 4\,mm^2$ piece of adhesive tape. Each tooth was mounted in a resin block. After resin dried, the tape was removed, leaving the exposed window. The labial surface of each tooth was polished using 500, 800, 1000, 2000, 3000, 4000, and 5000 grit sandpaper and then polished with alumina powder until the surface was glossy. An ultrasonic system was used to remove the residual enamel powder. Each enamel slab was sterilized with ultraviolet light. The initial surface hardness of each enamel slab was measured using a microhardness tester (FM-ARS 9000, Future-Tech Corp., Kanagawa, Japan) at four different points, each at least 100-µm apart, applying 50 g of force for 15 seconds. After surface hardness testing, each tooth was placed in a well of a 24-well polystyrene culture plate containing sterile pooled human saliva. The plate was then incubated at 37°C for 24 hours to allow the saliva to coat the enamel surface to obtain the glycoproteins from the saliva to initiate biofilm formation by *S. mutans*. After 24 hours, the saliva was removed, and *S. mutans* cultured in BHI broth with 0.5% sucrose was added to each well, and then plates were incubated in 5% CO_2 at 37°C for 24 hours. The 50 enamel slabs were then divided into five groups ($n = 10$) that were immersed in the test milk solutions: group 1: cow milk, group 2: unsweetened pistachio milk, group 3: sweetened pistachio milk, group 4 (positive control): 5% sucrose solution, and group 5 (negative control): deionized water. Each slab was immersed in its respective solution for 30 minutes every 4 hours, three times a day, for 5 days. After each immersion was complete, each tooth was rinsed twice in normal saline and placed back in its respective well containing artificial saliva. After 5 days, each tooth was briefly rinsed in PBS (pH 7.0). The surface microhardness of each slab was measured again and the % SHL was calculated as follows:

$$\%\ \text{surface microhardness loss} = \frac{\text{Initial SH} - \text{Final SH}}{\text{Initial SH}} \times 100.$$

Fluorescence Staining and Confocal Laser Scanning Microscope

One enamel slab from each test group was randomly selected at the end of 5 days for fluorescence staining. Each slab was briefly rinsed in PBS three times to remove the nonadhering cells. The biofilm on the enamel slab was stained with 3 µL 1:10 diluted SYTO-9 and propidium iodide (L7012 LIVE/DEAD BacLight Bacterial Viability Kit InvitrogenTM, Molecular. Probes Inc., OR, USA) and stored in the dark for 15 minutes, then analyzed with a confocal laser scanning microscope (CLSM; IX83ZDC, Olympus, Japan) using the live cell fluorescent imaging system. ALens at the power of 60× was used. Images of the biofilm thickness were obtained at 1-µm intervals at random positions of the slab. The images were processed using Stellaris8 software (Leica Microsystems, Germany).

Statistical Analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, United States). The Kolmogorov–Smirnov test was used to determine significant differences between *S. mutans* biofilm formation among the tested milk groups. For enamel demineralization, the data were presented as mean %SHL and significant differences were calculated using the analysis of variance (ANOVA) test and the post hoc test. A p-value < 0.05 was considered statistically significant.

Results

Amount of *S. mutans* Biofilm Formation

The OD ± standard deviation (SD) measurements for *S. mutans* biofilm formation in cow milk, lactose-free cow milk, goat milk, unsweetened pistachio milk, sweetened pistachio milk, and positive and negative controls were 0.082 (± 0.002), 0.086 (± 0.004), 0.083 (± 0.007), 0.0952 (± 0.010), 0.342 (± 0.072), 1.536 (± 0.084), and 0.071 (± 0.006), respectively (►**Fig. 1**). There was no significant difference in *S. mutans* biofilm formation among cow milk, lactose-free cow milk, goat milk, and unsweetened pistachio milk. The sweetened pistachio milk had significantly more biofilm formation than the other milk ($p < 0.05$). Thus, we used only the cow milk, unsweetened pistachio milk, and sweetened pistachio milk in the enamel demineralization experiments.

Enamel Demineralization

The %SHL ± SD for the cow milk, unsweetened pistachio milk, and sweetened pistachio milk, positive and negative controls were 20.01 (± 2.618), 22.088 (± 3.4), 35.49 (± 2.069), 52.984 (± 5.392), and 10.713 (± 2.495), respectively (►**Fig. 2**). The % SHL on the enamel slabs in sweetened pistachio milk was significantly higher ($p < 0.001$) compared with cow milk and unsweetened pistachio milk.

One enamel slab was randomly selected from each group to further visualize the enamel opacity and the area of surface hardness loss using a light microscope (10× magnification) after exposure to the test milk samples. Direct visualization revealed the enamel demineralization, which is the white opaque area in the middle of the slabs. The enamel demineralization area on the enamel slab in the 5% sucrose group (positive control) after an interval immersion for 5 days was noticeable. For the experiment groups, the sweetened pistachio milk group showed a larger enamel demineralization area on the enamel slab than the other test milk groups (►**Fig. 3**).

Fluorescence Staining and Confocal Laser Scanning Microscopy

Three-dimensional biofilm images were seen in ►**Fig. 4**. Live cells were stained and exhibited green fluorescence, whereas dead cells exhibited red fluorescence under CLSM. The

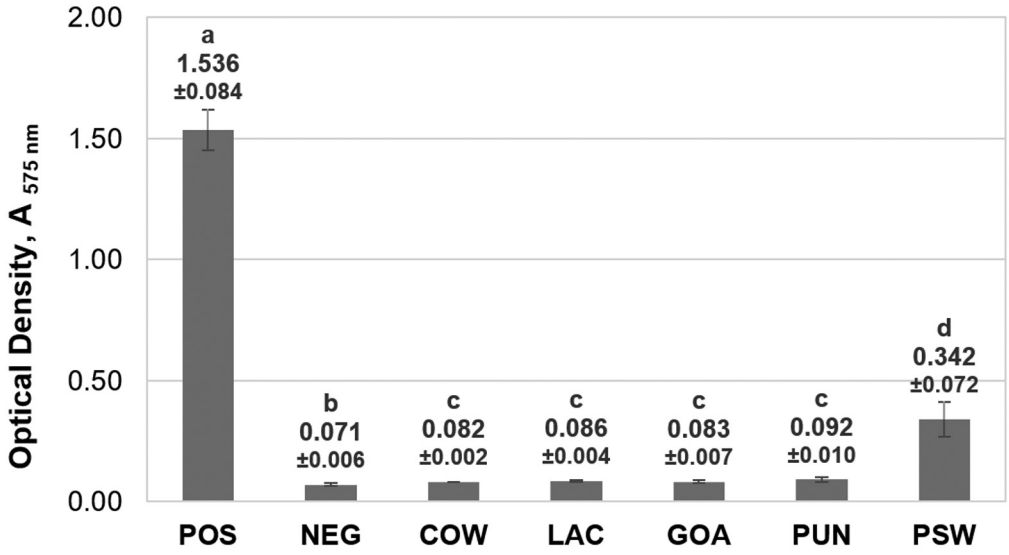

Fig. 1 Biofilm formation of *Streptococcus mutans* in the presence of various tested solutions. Data represent averages (±SD in error bars). COW, cow milk; GOA, goat milk; LAC, lactose-free milk; NEG, negative control (deionized water); POS, positive control (5% sucrose); PSW, sweetened pistachio milk; PUN, unsweetened pistachio milk. Note: Different superscripted letters ($^{a, b, c, d}$) represent statistically significant differences among treatments ($p < 0.05$ vs. all others).

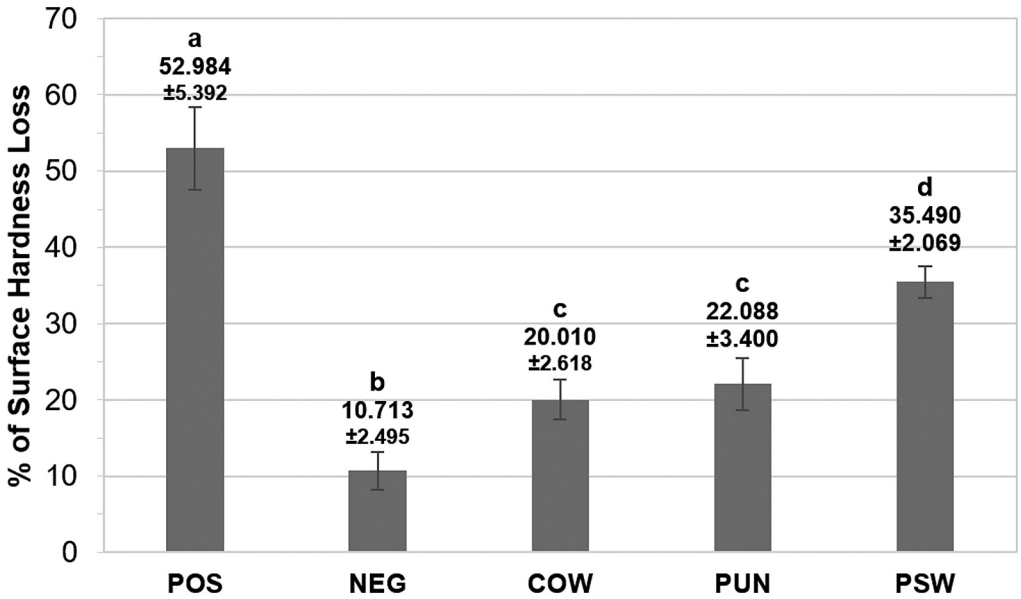

Fig. 2 Percentage of surface hardness loss (%SHL). Data represent averages (± SD in error bars). COW, cow milk; NEG, negative control (deionized water); POS, positive control (5% sucrose); PUN, unsweetened pistachio milk; PSW, sweetened pistachio milk. Note: Different superscripted letters ($^{a, b, c, d}$) represent statistically significant differences among treatments ($p < 0.001$ vs. all others).

positive control group (5% sucrose) contained the most live bacteria and had the highest *S. mutans* biofilm thickness. The negative control group (deionized water) exhibited the thinnest biofilm with numerous dead cells.

Biofilm formed in sweetened pistachio milk demonstrated a mushroom-like structure similar to the biofilm formed in the positive control, whereas cow milk and unsweetened pistachio milk had a flat biofilm architecture. The biofilm thickness in sweetened pistachio milk was thicker than the biofilm formed in the other milk. The live/dead cell ratio in biofilm exposed to unsweetened pistachio milk was comparable to those of cow milk. The highest percentage of *S. mutans* dead cells was detected in the unsweetened pistachio milk.

Discussion

Several studies have analyzed the cariogenic properties of cow milk by comparing plain and sweeten cow milk.[14,21,22] Also, previous studies have compared the effect of plant-based milk and cow milk on *S. mutans* biofilm formation.[23,24] They found that plant-based milk produced less

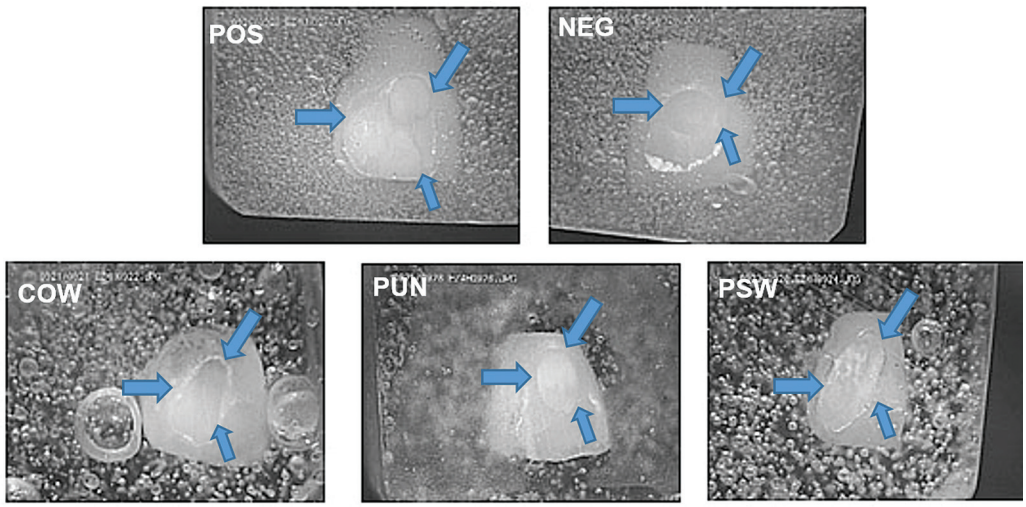

Fig. 3 Representative images of enamel surface after being treated with various solutions for 5 days. COW, cow milk; NEG, negative control (deionized water); POS, positive control (5% sucrose); PSW, sweetened pistachio milk; PUN, unsweetened pistachio milk.

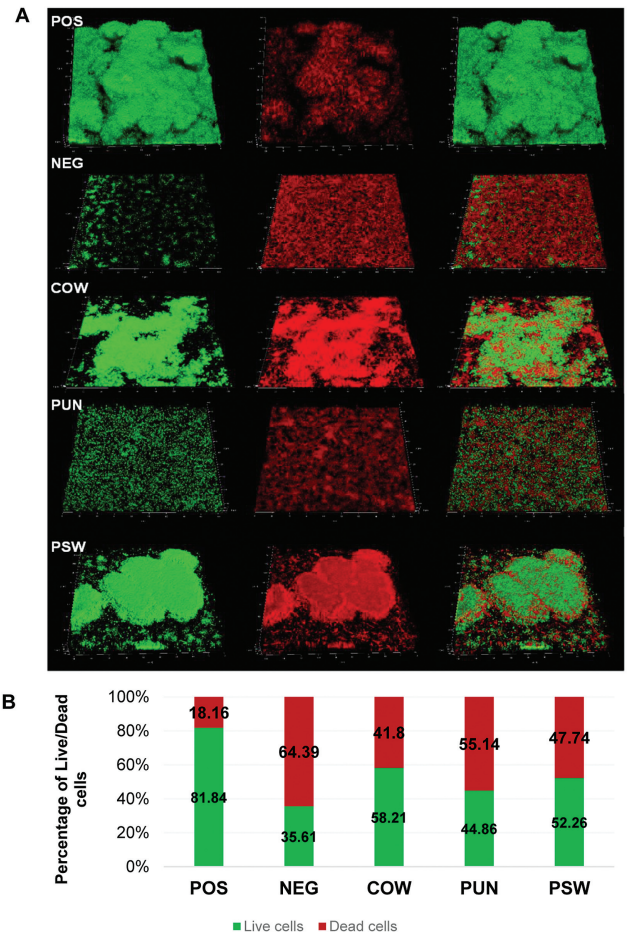

Fig. 4 (A) *Streptococcus mutans* biofilm on enamel slab of the primary teeth visualized using CLSM. (B) Percentage of *S. mutans* live and dead cells in biofilms on enamel slab using CLSM. Green indicates live cells, and red indicates dead cells. CLSM, confocal laser scanning microscope; COW, cow milk; NEG, negative control (deionized water); POS, positive control (5% sucrose); PUN, unsweetened pistachio milk; PSW, sweetened pistachio milk.

biofilm but had a poorer buffering capacity compared with cow milk. When sugar was added to plant-based milk, it resulted in greater biofilm formation compared with cow milk. However, no study is currently evaluating biofilm formation between cow milk and goat milk together with plant-based milk. In our study, there was no significant difference in the levels of biofilm formation in cow milk, lactose-free cow milk, goat milk, and the unsweetened pistachio milk. The sweetened pistachio milk that contains 3.3% sucrose had significantly more *S. mutans* biofilm formation than the other milk samples. Our findings were similar to previous studies that demonstrated when sucrose was added in the plant-based milk, it was associated with a large amount of EPS production, which resulted in more biofilm formation.[13,20,25] We selected to compare *S. mutans* biofilm formation between the sweetened and unsweetened pistachio milk because some plant-based milk such as soy milk is potentially cariogenic even without adding sucrose and was able to support significant biofilm growth.[13] Another study found that chocolate cashew milk resulted in greater *S. mutans* biofilm formation than unsweetened cashew, coconut, and flax milk.[15] Moreover, when using CLSM to analyze the biofilm thickness and structure, *S. mutans* biofilm formed in the presence of the sweetened pistachio milk exhibited the thickest amount of biofilm, with the bacteria embedded in a matrix primarily composed of EPS, along with a signature mushroom-like structure compared with the other milk samples that had a flat biofilm structure. Consistent with a previous study using CLSM, their results indicated that when biofilm was formed in a sucrose-rich solution, it demonstrated many bacterial cells in a cluster of biofilm growth with a well-developed EPS matrix, enhancing the bulk, volume, and integrity of the biofilm matrix.[26] Furthermore, in this study, *S. mutans* biofilm formed on enamel slabs in the 5% sucrose contained the most live bacteria and had the highest biofilm thickness, while deionized water group exhibited a thin biofilm with numerous dead cells. The live/dead cell ratio in biofilm exposed to unsweetened pistachio milk was comparable to those of cow milk. These results demonstrate that *S. mutans* survives better in nutrient-rich environments.

Although adding sucrose in plant-based milk increased *S. mutans* biofilm formation, other factors might play a role in biofilm formation such as bioactive compounds in plants which might feature antibacterial activity. Recent studies evaluated whether or not various alternative milks, such as cashew milk, almond milk, coconut milk, flax milk, macadamia milk, and pecan milk, enhanced *S. mutans* biofilm formation. Their results demonstrated that *S. mutans* biofilm formation was lowest in unsweetened flax milk. They suggested that the low *S. mutans* biofilm formation might be due to the absence of added sugar and the presence of flax seeds that are rich in omega-3 fatty acids and phytochemicals with strong antimicrobial effects.[15] Likewise, in this study, *S. mutans* biofilm formation was low in unsweetened pistachio milk. This might be not only due to the absence of added sugar but also due to the presence of pistachio nut components. Pistachio nuts (*Pistacia vera* L.) produce oleoresins comprising different bioactive compounds, such as triterpenes and essential oils.[27] A study showed that even a very low concentration of the oleoresin from *P. vera* L. possesses antibacterial properties against *S. mutans* (ATCC 25175) that limits the bacteria's ability to form biofilm.[28]

Lactose in cow milk is less cariogenic than sucrose and is additionally not a favored substrate for *S. mutans* in producing EPS, while sucrose is essential. This is the reason sucrose has been considered the most cariogenic carbohydrate.[29] *S. mutans* can also metabolize lactose and produce acid. However, a study demonstrated that lactose-supplied bacterial biofilm had lower EPS than sucrose-supplied biofilm.[21] The amounts of lactose in cow milk, lactose-free cow milk, and goat milk did not significantly enhance *S. mutans* biofilm formation.

In this study, when visualizing the biofilm structure on the enamel slabs immersed in cow milk using CLSM, the biofilm structure was flat. A previous study showed that the polysaccharide formed by *S. mutans* in the presence of lactose was different from sucrose due to the complexity of the lactose metabolism pathway, resulting in less biofilm formation.[30] Our findings are consistent with previous studies where *S. mutans* biofilm produced by lactose-free cow milk was similar to those exposed to cow milk.[21,22]

The basic composition of cow milk and goat milk is relatively similar. However, goat milk has a higher content of total protein, fats, and minerals than cow milk, with the similar lactose amount (4.11 and 4.47%, respectively).[16] This study found no significant difference in *S. mutans* biofilm formation among goat milk, lactose-free cow milk, and cow milk.

Many studies evaluated the effects of milk on the enamel demineralization, but most of them were conducted on permanent or bovine teeth.[13,18] This is the second study to examine the effect of a plant-based milk (pistachio milk) on the enamel demineralization of primary incisor teeth. Our previous study found a significantly greater %SHL with the sweetened plant-based milks (almond and soy milk) than cow milk.[20] In this study, the highest %SHL was found in the enamel slabs immersed in the sweetened pistachio milk compared with cow milk and unsweetened pistachio milk. Previous study showed similar result regarding the effect of cow milk on bovine teeth, indicating that its %SHL was not significantly different from the negative control.[31] In addition, we present an enamel demineralization or the early stage of enamel caries that typically presents clinically as a white opaque area called a white spot lesion in the enamel slabs of both the positive control (5% sucrose) and the sweetened pistachio milk groups. When measuring the surface microhardness of the enamel slabs in the sweetened pistachio milk group after 5 days of immersion, the mean surface hardness value was $228.847 \pm 20.560\ \mathrm{kg/nm^2}$, which was similar to the score of code 1 by the International Caries Detection and Assessment System (ICDAS) that states that code 1 is a visible noncavitated lesion seen when dry.[32,33] Furthermore, the enamel slabs that were immersed in 5% sucrose showed enamel opacity throughout the window of the enamel slab with mean surface hardness value of $164.156 \pm 16.415\ \mathrm{kg/nm^2}$, which is associated with ICDAS code 2 (distinct visual change in enamel).[32,33] In contrast, the enamel slabs immersed in cow milk, unsweetened pistachio milk, or deionized water all demonstrated mean surface hardness values higher than sweetened pistachio milk. Several studies reported that the addition of sucrose to milk enhanced enamel demineralization.[25,34]

This result is interesting because this is the first study to represent the effects of cow milk and alternative milks on the enamel demineralization of human primary teeth in relevant to the ICDAS system that currently used in clinical practice. Furthermore, a previous study reported that the biofilm architecture was associated with cariogenic conditions. They observed that the white spot lesion matched the mushroom-shaped biofilm communities, resulting in deeper demineralization and more integrated mineral loss than flat-shaped biofilm.[35] Because EPS contributes to a decrease in environmental pH, the surface hardness loss was more pronounced in sweetened pistachio milk than those formed in the no sugar added groups.[29] In contrast, the present study showed that the %SHL of the enamel slabs immersed in the unsweetened pistachio milk were not significantly different from those of cow milk. The cariostatic properties of cow milk have been well known from several studies. The high concentration of calcium and phosphate present in cow milk may contribute to a decrease in enamel demineralization.[36] Moreover, casein, the main protein found in milk, may contribute to stabilize the high calcium phosphate concentration in milk, avoiding its precipitation. In addition, proteins may provide an acid-buffering capacity to the milk, which is also relevant for reducing demineralization.[31,33] Although the plant-based milk from nuts is low in calcium and protein, a study found that pistachio milk has a higher concentration of fat, a cariostatic factor, than cow milk.[36]

The limitation of this study is that a single species bacterial biofilm may not represent the dynamic and structurally complex multispecies dental plaque formed in the oral cavity. Further study with a multispecies biofilm is strongly recommended. Other factors that involved in cariogenic properties in pistachio milk such as buffer capacity

and pH levels would be better to obtain and lead to further knowledge.

In conclusion, this study showed that *S. mutans* biofilm formation was significantly higher when formed in the presence of sweetened pistachio milk giving the thickest biofilm and demonstrated a signature mushroom-like structure when using CLSM. In contrast, cow milk and unsweetened pistachio milk yielded flat biofilm. The sweetened pistachio milk caused greater enamel demineralization, forming white spot lesion, than cow milk and unsweetened pistachio milk. These results suggest that the added sugar in pistachio milk results in higher cariogenic properties regarding the amount of biofilm formation by *S. mutans*, structure of the biofilm formed, and the ability to demineralize enamel in human primary teeth.

Ethical Consideration

This study was approved by the Ethics Approval Review Board, Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University (MU-DT/PY-IRB 2020/015.2205).

Conflict of Interest

None declared.

Acknowledgments

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