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Effectivity of Probiotic and Non-Probiotic Milk Consumption on Salivary pH and *Streptococcus mutans* Count

Siska Ella Natassa[1,2], Sondang Pintauli[2], Syafruddin Ilyas[3]
[1]*Universitas Sumatera Utara, Indonesia, Magister Degree Programme, Faculty of Dentistry*
[2] *Universitas Sumatera Utara, Indonesia, Faculty of Dentistry, Department of Preventive Dentistry*
[3] *Universitas Sumatera Utara, Indonesia, Faculty of Mathematics and Natural Sciences, Department of Biology*

Abstract: *Probiotic is a living microorganism confer a health benefit including oral health benefits by producing bacteriocin. This study aims to analyze the salivary pH difference and Streptococcus mutans count before and after consumption of probiotic and non-probiotic milk. The study was conducted with 60 students aged 12-15 years old, randomly classified to two groups by purposive design. Test group consisted of 30 students consuming probiotic milk and the other 30 consuming non-probiotic milk. Saliva samples were collected by spitting method at baseline, 3 days and 7 days after the intervention. Salivary pH was measured using a digital pH meter. Streptococcus mutans was cultured in TYCSB media and incubated in an anaerobic jar for 2x24 hours. The mean salivary pH before consumption of probiotic milk was 6.59±0.35 which increased to 6.73±0.24 and 7.14±0.21, as for non-probiotic milk, 6.64±0.49 to 6.58±0.28 and 7.17±0.28. The mean Streptococcus mutans count before probiotic milk consumption was 53.07±5.55 CFU/ml which reduced to 29.13±02.20 and 6.67±02.88, whereas for non-probiotic milk, 48.73±8.12 CFU/ml decreased to 31.7±02.50 and 14.7±01.91. Probiotic milk effectively increases salivary pH as compared to non-probiotic milk on the third day after consumption (p=0,005) with a statistically significant reduction in Streptococcus mutans colony count on the third and seventh day after consumption (p=0,005). The use of probiotics in dentistry has high potential in increasing salivary pH and inhibiting Streptococcus mutans growth, thus eliminating the risk factors of dental caries.*

Keywords: *non-probiotic, probiotic milk, salivary pH, Streptococcus mutans*

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I. Introduction

Caries is a multifactorial disease that stems from bacteria causing demineralization in the dental enamel.[1,2] This is triggered by a continuous interaction between cariogenic microorganisms (especially *Streptococcus mutans*), carbohydrate-rich-diet and host factors such as salivary flow and buffer capacity.[3] *Streptococcus mutans* attachment in tooth surface. This may subsequently lead to an acidogenic environment of oral cavity with low pH (less than 5.5) and rapid metabolism of carbohydrates.[4] Many studies reported various preventive approaches on dental caries, among them, conventional preventions such as prophylaxis and fluoride application, caries risk assessment, and consumption of non-cariogenic food and beverages, such as probiotics.[4] Probiotics are essential to oral health in preventing plaque formation, eliminating halitosis and reducing caries risk.[5] Probiotic strain can be added to a variety of food and beverages, one of which includes milk. Milk contains calcium which remains the primary mineral source for bones and teeth. Milk contains casein phosphopeptide that has a prime role in oral health for its inhibitory effects in demineralization and stimulating remineralization of the teeth.[6] Furthermore, probiotic products are commercially available in the forms of tablets, candies, ice cream, yogurt, cheese and powders. Consumption of probiotic products is a necessary approach in preventing dental caries by inhibiting the growth of pathogenic bacteria and stimulate salivary secretion, thus maintaining the microbial balance in oral cavity.[7] Srivastava, et al reported that consumption of drinks that contain probiotics routinely for 7 days can decrease salivary *Streptococcus mutans* count before consumption which was 4.46±0.3 to 4.24±0.3 and increase salivary pH before consumption was 7.35±0.42 to 7.58±0.4.[8] This study is appropriate to Javid, et al who reported the reduction of *Streptococcus mutans* count after 2 weeks of consumption probiotic which was statistically significant (p<0.001). Their study revealed that the mean *Streptococcus mutans* count before consumption was 2624±137.73 and reduced to 621±69.99, after consumption.[4] This study aims to analyze the salivary pH difference and *Streptococcus mutans* count before and after consumption of probiotic and non-probiotic milk.

II. Materials and Methods

This was a quasi-experimental study with pretest-posttest control group design This study was conducted on students aged 12-15 years at Madrasah Tsanawiyah Ta'dib Al Muallimin Al Islamy school. Samples were selected purposively based on the following criteria: having at least 2-5 superficial caries, no habit of smoking, never used a mouthwash, no habit of brushing teeth with *siwak,* and no history of allergy milk and other dairy products.

Total samples were 60 children were selected then indiscriminately divided into two groups of 30 each. Group A subjects were consuming 180 ml UHT milk with added probiotic, whereas group B subjects were given only UHT milk. Subjects were instructed not to consume any food or beverages for at least an hour before consuming the allocated probiotic and non-probiotic milk. Salivary samples were collected thrice by spitting method at baseline, 3 days and 7 days after intervention at 09.00-10.00am. The spitting method was applied in this study due to the higher salivary production compared to other methods. Saliva samples were then brought to the microbiology laboratory in the Medical Faculty of Universitas Sumatera Utara to measure salivary pH and *Streptococcus mutans* count. Salivary pH measurement was performed using a digital pH meter.

Streptococcus mutans was cultured in TYCSB (Tryptone Yeast Cystine Sucrose Bacitracin) selective media by mixing 249.97 grams of powder in 1000 ml distilled water and boiled. Media was sterilized using autoclave with a pressure of 15 atm (121^{o}C) for 15 minutes and cooled down to 45-50^{o} and subsequently poured into a sterile petri dish. Media was then cooled down at room temperature. Small samples of saliva were then taken using a micropipette as much as 0.5 ml, diluted 4 times. At the fourth dilution, 0.1 ml of the saliva sample was taken and poured into the media before being placed in an anaerobic jar (anaerobic conditions, 10% H_2, 10% CO2 and 80% N_2) then incubated at 37^{o}C for 2x24 hours. Later, the number of colony forming units (CFU / ml saliva) was counted, twice. The final amount of *Streptococcus mutans* colonies was obtained by calculating the mean values of the two measured results and the morphology was identified using a light binocular microscope with 1000x magnification. Data were analyzed with *Generalized Linear Model Repeated Measured* and *Simple Linear Regression Test*. Ethical approval was obtained from Health Research Ethical Commitee Medical Faculty of Universitas Sumatera Utara. The proper information regarding the purpose of the study was given to the samples and asked to be filled by them.

III. Results

The results showed that the research subjects were mostly 15 years old (36%) and males (63.3%) with an average of two teeth with superficial caries (61.7%).

In the probiotic milk group, the mean salivary pH before consumption was 6.59±0.35 whereas, in the non-probiotic group, it was 6.64±0.49. Statistical analysis results showed that salivary pH of both groups had no significant difference (p= 0.668) (Table 1).

The mean *Streptococcus mutans* count in the probiotic group before consumption was 52.26±11.86 CFU/ml whereas in the non-probiotic group was 48.70±8.12 CFU/ml. There was no significant difference in both groups (p=0.115) (Table 1).

Table 1. Mean salivary pH and *Streptococcus mutans* count before consuming probiotic and non-probiotic milk (n=60)

Group	Mean Salivary pH ($\overline{X}$ ± SD)	*p*	Mean *Streptococcus mutans* count (CFU/ml)	*p*
Probiotic milk	6.59±0,35	0.668	52.26±11.86	0.115
Non Probiotic milk	6.64±0,49		48.70±08.12	

The result of this study showed an increased salivary pH and decreased *Streptococcus mutans* count from 0 day to 3 and 7 days after milk (probiotic and non-probiotic) consumption which was 6.61 ± 0.43 increased to 6.65 ± 0.27 and 7.15 ± 0.24, as for *Streptococcus mutans* count, there was a reduction from 50.90 ± 10.64 to 30.42 ± 2.67 and 10.68 ± 4.72 (Table 2).

Table 2. Mean salivary pH and *Streptococcus mutans* count after consuming probiotic and non-probiotic milk (n=60)

Mean (X ± $\overline{SD}$)	Observation Time		
	0 Day	3 Days	7 Days
Salivary pH	06,61±0,43	06,65±0,27	07,15±0,24
Streptococcus mutans	50,9±10,64	30,42±2,67	10,68±4,72

In the probiotic group, there was increased salivary pH and decreased *Streptococcus mutans* count on day 0 to day 3 and 7 after probiotic milk consumption which was 6.59±0.36 increased to 6.73±0.24 and 7.14±0.21, and *Streptococcus mutans* count from 53.07±12.43 decreased to 29.13±2.20 and 6.67±2.88. In the non-probiotic group, the salivary pH decreased on day 0 to the third day which was 6.64±0.49 to 6.58±0.28 and increased to 7.17±0.28 on the seventh day after consumption, while *Streptococcus mutans* count reduced from 48.73±08.12 to 31.70±02.50 and 14.70±01.91 (Table 3).

Table 3. Mean salivary pH and *Streptococcus mutans* count (n=60)

Time Consumption (Day)	Probiotic		Non-Probiotic	
	Mean pH ($\overline{X}$ ± SD)	Mean *S. mutans* ($\overline{X}$ ± SD) CFU/ml	Mean pH ($\overline{X}$ ± SD)	Mean *S. mutans* ($\overline{X}$ ±SD)CFU/ml
0	6,59±0,36	53,07±12,43	6,64±0,49	48,73±08,12
3	6,73±0,24	29,13±02,20	6,58±0,28	31,70±02,50
7	7,14±0,21	06,67±02,88	7,17±0,28	14,70±01,91

Based on the *Generalized Linear Model Repeated Measured* test, there was a statistically significant difference in mean salivary pH on day seven between the two groups (probiotic and non-probiotic milk) (p = 0,000), while *Streptococcus mutans* count was statistically significant reduced on day three and seven (p = 0,000) (Table 4).

Table 4. Effectivity of milk (Probiotic and Non-Probiotic) Consumption on salivary pH and *Streptococcus mutans* based on time (n=60)

Time Consumption (Day)	Mean salivary pH ($\overline{X}$ ± SD)	*p*	Mean *Streptococcus mutans* ($\overline{X}$ ± SD) CFU/ml	*p*
0 - 3	(6,61±0,43) – (6,65±0,27)	0,308	(50,90±10,64) – (30,42±02,67)	0,000*
3 - 7	(6,65±0,27) – (7,15±0,24)	0,000*	(30,42±02,67) – (10,68±04,72)	0,000*

*p<0,05

In the probiotic group, there was a statistically significant difference in salivary pH between day 0 and 3, and day 3 and 7, with a value of p = 0.006 and p = 0.000, respectively. Meanwhile, in the non-probiotic group, there was a difference in mean salivary pH only between days 3 and 7 (p = 0,000) (Table 5).

Table 5. Effectivity of Probiotic and Non-Probiotic Milk consumption on mean salivary pH

Time Consumption (Day)	Probiotic		Non-Probiotic	
	Mean pH ($\overline{X}$ ± SD)	*p*	Mean pH ($\overline{X}$ ± SD)	*p*
0 - 3	(6,59±0,36)-(6,73±0,24)	0,006*	(6,64±0,49)–(6,58±0,28)	0,373
3 - 7	(6,73±0,24)–(7,14±0,21)	0,000*	(6,58±0,28)–(7,17±0,28)	0,000*

*p<0,05

There was a statistically significant reduction in *Streptococcus mutans* count before and after milk consumption in both groups p=0,000 (Table 6).

Table 6. Effectivity of probiotic and non-probiotic milk on *Streptococcus mutans* count

Time Consumption (Day)	Probiotic		Non-Probiotic	
	Mean *S. mutans* ($\overline{X}$ ± SD)	*p*	Mean *S. mutans* ($\overline{X}$ ± SD)	*p*
0 – 3	(53,07±12,43)–(29,13±02,20)	0,000*	(48,73±08,12)–(31,70±02,50)	0,000*
3 - 7	(29,13±02,20)–(06,67±02,88)	0,000*	(31,70±02,50)–(14,70±01,91)	0,000*

*p<0,05

Based on Simple Linear Regression Analysis, the probiotic milk is greatly effective in increasing salivary pH compared to non-probiotic milk after three days of consumption. Similarly, probiotic milk is more effective in decreasing *Streptococcus mutans* count after three and seven days of consumption (p<0.05) (Table 7 & 8).

Table 7. Comparison of Salivary pH Between Probiotic and Non-Probiotic Milk Group

Mean Difference Salivary pH	Group			
	Probiotic and Non-Probiotic Milk			
	A	*B*	R^2	*p*
0 day – 3 day	-0,097	0,229 x_0,x_1	0,129	0,005*
3 day -- 7 day	0,554	-0,107 x_0,x_1	0,038	0,136

*a=intercept; b=treatment score; R=determined coefficient; x0=probiotic group; x1=non-probiotic; p<0,05

Table 8: Comparison of *Streptococcus mutans* count between Probiotic and Non-Probiotic Group

Mean Difference *Streptococcus mutans* Count	Group			
	Probiotic and Non-Probiotic Milk			
	A	***B***	R^2	***p***
0 day – 3 day	16,208	$7{,}125x_0{,}x_1$	0,111	0,009*
3 day -- 7 day	17,083	$4{,}417x_0{,}x_1$	0,038	0,000*

*a=intercept; b=treatment score; R=determined coefficient; x_0=probiotic group; x_1=non-probiotic; p<0,05

IV. Discussion

There was no significant difference in mean salivary pH and number of *Streptococcus mutans* between the probiotic and non-probiotic milk groups before consumption with a value of p>0.05. This shows that there were similar initial conditions between the two groups (baseline). This was substantiated by the fact that the initial conditions were all under control prior to the study whereby subjects were instructed not to consume any food or beverages for at least an hour before. In addition, subjects in both groups were carefully preselected to include only those with superficial caries affecting at least 2 to 5 teeth (Table 1).

The mean salivary pH increased after seven days of consuming probiotic and non-probiotic milk, whereas the *Streptococcus mutans* count had decreased. Milk is rich with antioxidant, anti-cariogenic, and antibacterial content which impedes the breakdown of carbohydrates into lactic acid and aspartic acid by bacteria leading to increased salivary pH. Furthermore, milk has a high buffer capacity, despite the collection of saliva was conducted 10 minutes after consumption.[9] Following consumption, carbohydrates are suspected to produce acid through the glycolysis process and consequently leads to decreased salivary pH below to critical pH. According to Stephan's curve, after consuming carbohydrates, the salivary pH will decrease within 5-10 minutes and require approximately 30-60 minutes to return to normal pH by the salivary buffer system. For this reason, increased salivary buffer capacity would produce a stable pH.[10]

The antibacterial effect of milk can eliminate *Streptococcus mutans* by inhibiting adhesion to the tooth surface thereby obstruct the formation of biofilm.This may be due to milk having vital components that shield against caries, especially calcium, phosphorus and buffer capacity associated with anti-cariogenic and antibacterial activities.[6,11]

The results of the study in the probiotic milk group showed an increased mean salivary pH before consumption from 6.59±0.35 to 6.73±0.24 and 7.14±0.21 on the third and seventh day, respectively. In the non-probiotic milk group, the mean salivary pH decreased from 6.64±0.49 to 6.58 ± 0.28 before consumption and on the third day but increased to 7.17 ± 0.28 on the seventh day after consumption (Table 2). This may be caused by the incomplete breakdown of carbohydrates into lactic and aspartic acid on the third day leading to reduced production of ammonia base substances from the milk solution. The stable pH in the probiotic milk group may be caused by strains of probiotic bacteria that interfered and replaced the pathogenic bacteria colony to a more beneficial bacteria colony by producing less organic acid thus preserving the salivary pH in an alkaline environment.[11]

The mean *Streptococcus mutans* count of the probiotic milk group decreased from 53.07±12.43 CFU/ml to 29.13±2.2 CFU/ml and 6.67±2.88 CFU/ml. There was also a noteworthy reduction in the non-probiotic milk group from 48.73±8.12 CFU/ml to 31.70±2.5 CFU/ml and 14.7±1.91 CFU/ml. The number of *Streptococcus mutans* decreased in the probiotic milk group because of non-pathogenic probiotic strains such as *Bifidobacterium* and *Lactobacillus acidophilus,* which had inhibited the growth of pathogenic bacteria by producing bacteriocins. This event was also further aided by the combination of local and systemic immune system, and the natural body defense mechanism.[12] Bacteriocin is an antimicrobial peptide compound with a low molecular weight in the form of a short peptide protein chain which can inhibit or even eradicate microbes, by damaging the structure of *Streptococcus mutans* bacterial cell membranes. Bacteriocin can occupy and adhere to various cells in the oral cavity and produce a lanbiotic type of bacteriocin that greatly benefits gram-positive bacteria, consequently leading to a decline in gram-negative bacteria population.[13]

Based on the time of milk consumption, there was a statistically significant increment in salivary pH on days 3 and 7 (p = 0,000) (Table 4). This occurred due to the anti-cariogenic and antibacterial content in the milk which had significantly reduced both the oral bacteria colony and carbohydrate breakdown activity into acid after seven days of consumption. For that reason, the subjects' salivary pH value had increased to alkaline conditions.[6]

The probiotic milk group had significant differences in salivary pH before consumption compared to the third day and the third day compared to the seventh day after consumption, each with a value of p<0.05. However, in the non-probiotic group, the salivary pH was significantly different on the third day compared to the seventh day after milk consumption (Table 5). Probiotic strains contain lactic acid-producing bacteria which secrete several metabolites that function as antimicrobials. For instance, acetic and propionic acids as by-products of hetero-fermentative fermentation by probiotic strains interact with the bacterial cell membrane and causes intracellular acidification and protein denaturation.[13]

Table 4 shows a significant difference in mean values of *Streptococcus mutans* count between day 0, day 3, and day 7 after milk consumption ($p<0.05$). Milk has beneficial biological functions and high mineral content that can affect the metabolism of *Streptococcus mutans* by inhibiting and restricting its viability in an acidic environment (pH <5.5).[11] Milk and saliva have similar biological constituents, such as lactose, fat, caseins, and lactalbumins (only in milk), and glycoprotein340 which is derived from the innate immune system (only in saliva). Generally, consuming milk can considerably reduce the acidification period and subsequently preserve the salivary pH in an alkaline condition.[13]

There was a statistically significant reduction in *Streptococcus mutans* count before and after milk consumption in both the probiotic and non-probiotic milk groups ($p=0.000$) (Table 6). Probiotic and non-probiotic milk possess an anti-cariogenic agent capable of preventing caries. *Streptococcus mutans* is a bacterium in the oral cavity that produces bacteriocin which is associated with the onset of caries. The decline of *Streptococcus mutans* count in saliva occurred due to the possibility of the anti-cariogenic activity in milk which is associated with its high amounts of calcium, phosphate and milk protein.[11-3] Proteins in milk, such as α, β, ꝁ-casein, and lactoferrin can inhibit the proliferation of *Streptococcus mutans* by binding the bacteria to salivary receptors located within the hydroxyapatite layer. Peptides derived from Ꝁ- and β-casein, such as casein glycomacropeptide (CGMP) and caseinphosphopeptide (CPP), also bind to the salivary pellicle by displacing albumin which promotes adhesion of *Streptococcus mutans*. In some reports, milk and Ꝁ-casein are capable of reducing the rate of glucosyltransferase (GTF) absorption into the teeth. In this context, it is important to understand that glucosyltransferase is a polysaccharide that produces an enzyme similar to a derivate of *Streptococcus mutans* and converts disaccharides (especially sucrose) to glucans. Extracellular glucan is a structural component of the dental plaque matrix, which merges the entire plaque mass, enabling it to attach on the dental surface.[6,14]

Probiotic as effective as non probiotic milk in increasing salivary pH after seven days of consumption ($p=0,136$). This may be caused the increasing of salivary pH in milk group after seven days consumption. Milk contains of casein that produced peptides and amino acids, which catabolism them further. Its can raise the pH and prevent demineralization. This process may neutralize acid production from lactose fermentation and preserving alkaline environment in oral cavity.[11] The probiotic milk is greatly effective in increasing salivary pH compared to the non-probiotic milk after three days of consumption. It is also effective in decreasing *Streptococcus mutans* count after three and seven days of consumption ($p<0.05$) (Table 7&8). This can be attributed to the tendency of probiotic bacteria to stabilize the pH of the oral cavity after consumption as this would maintain homeostasis and affect the composition of saliva, such as the concentration of mucins and salivary immunoglobulins, as reported in an *in vivo* study. In addition, probiotics also play a valuable role in stimulating fluid production within the body. Probiotics have the ability to alter the epithelial cells of the parotid glands to produce more beta-2 adhesions in saliva which can increase salivary secretion.[15] It is also worth mentioning that probiotics contain bacteria that do not ferment lactose and sucrose, and can dominate the oral cavity thus hinder the colonization of *Streptococcus mutans* on the tooth surface.[8]

Probiotic bacteria in the oral cavity function through either direct or indirect mechanisms. The direct mechanism is associated with its ability to bind to plaques or biofilms through protein bonding. Probiotic bacteria will compete with pathogenic bacteria within the plaque and its other derivatives would inhibit the binding of other oral pathogens onto the plaque surface. These produced substances are better known as antimicrobial peptides or proteins called bacteriocins and are fully equipped to attack other pathogens in the same ecological environment.[13] The indirect mechanism of probiotics involves local and systemic immunity and the production of antioxidants.[12] In general, the indirect mechanism of probiotics is to prevent or treat oral diseases by modulating inflammatory bacteria, inhibiting plaque formation, and reducing the number of disease-causing microorganisms.[12] With this mechanism, the effects of pathogenic bacteria will be minimized by probiotic bacterial activity. Probiotic bacteria cannot colonize and attach permanently to the oral cavity, thus a continuous administration is needed. [8]

Bifidobacterium bifidum and *Lactobacillus acidophilus* act as immunomodulators mainly in increasing the number of secretory IgA (sIgA) producing cells that are only found in saliva and other immunoglobulin-producing cells, as well as stimulate the release of local interferons which facilitate antigen transport. sIgA is a dominant immunoglobulin in saliva and primarily functions to provide protection against cariogenic microorganisms as the first line of defense against invading pathogens within the oral environment. It was also reported that sIgA in saliva can inhibit the onset of *Streptococcus mutans* adhesion to the salivary pellicle on the tooth surface. Besides, sIgA is capable of inhibiting the function of the glucosyltransferase enzyme from *Streptococcus mutans.* On a different note, *Lactobacillus acidophilus* is able to compete with other pathogenic bacteria by preserving a homeostatic environment by means of producing DL-Lactic acid which is the formation of lactic acid and amylase that aids the growth of other probiotic bacteria.[13]

Further research is required regarding the effect of consuming probiotic milk on salivary pH and *Streptococcus mutans* count using different bacteria strains of probiotic. The results of this study indicate that probiotic bacteria can increase salivary pH and provide an antagonistic effect on *Streptococcus mutans*. To intensify the effect of probiotic bacteria on *Streptococcus mutans*, probiotic milk should be consumed in the long term as the bacteria can only last for 1 week after ceasing consumption.[8]

V. Conclusion

Probiotic milk is greatly effective in increasing salivary pH and reducing *Streptococcus mutans* count compared to non-probiotic milk.

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