Evaluation of Probiotic Milk on Salivary Mutans Streptococci Count: An In Vivo Microbiological Study

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Objective*- The objective of the present study was to investigate the effect of probiotic Lactobacillus casei Shirota on the values of mutans streptococci counts in saliva.* ***Study design****- 31 children were included in this double blinded study. Study design included 4 periods which consisted of a run in period, two intervention periods and a washout period. During the intervention periods children were either given probiotic milk or control milk for 10 days. Pre and post intervention salivary samples were subjected to microbiological evaluation. Then numbers of mutans streptococci were taken by identifying colony morphology.* ***Results*** *- A statistically significant reduction of mutans streptococci colony count was noted in the probiotic group (p=.003). The reduction in children with higher levels of mutans streptococci (10^5) after intervention was 34% in the probiotic group.* ***Conclusion****- Daily consumption of milk containing probiotic bacteria can reduce the levels of mutans streptococci and may contribute to the prevention of dental caries.*

Key words*: caries, school children, mutans streptococci, Lactobacillus casei Shirota.*

INTRODUCTION

Dental caries is one of the most common preventable childhood diseases which is often not self-limiting and without intervention can progress until the tooth is destroyed. At present, an ecological plaque hypothesis is believed to explain the aetiology of dental caries that reconciles the key elements of the earlier hypothesis. Key features are that the selection of "pathogenic" bacteria is directly coupled to changes in the environment and diseases need not have a specific etiology. In addition, any species with relevant traits can contribute to the disease process.[1]

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Currently, most commonly used chemo-prophylactic agents have the disadvantage that they inhibit bacterial growth and biofilm formation, wiping out the oral flora which may lead to unwanted consequences.[2] While a number of medicaments can suppress pathogenic microbiota, none of them have been able to successfully prevent the regrowth of residual pathogen.[3] Selective inhibition of pathogenic microorganisms can be achieved by the use of selective probiotic strains.

The concept of probiotics evolved from Elie Metchnikoff's (1907) idea that the bacteria in fermented products could compete with microbes that are injurious to the host and thus are beneficial for health.[4] In the past several years, probiotics have increasingly been investigated from the oral health perspective, and the probiotic approach has been a popular method for modulating microbial communities.[5]

Dairy foods such as yogurt, milk, and cheese are considered useful vehicles for probiotic delivery, although the best way of administration has yet to be identified.[6] It is suggested that the vehicle for administration should be of milk origin due to presence of casein phosphopeptides that have an effect on demineralization and promote remineralization of dental caries.[7] Various bacterial strains which have been successfully used as an antimicrobial agent against mutans streptococci are *Lactobacillus rhamnosus GG*, *Lactobacillus reuteri* ATCC 55739, *Lactobacillus reuteri* CF2-7F, *Lactobacillus reuteri* MF2-3, *Lactobacillus reuteri* FJ1 and *Bifidobacterium* DN-173010.[2] Using randomized controlled trials, Meurman and colleagues demonstrated that long-term consumption of milk containing the probiotic *Lactobacillus rhamuosus* GG strain reduced initial caries in kindergarten children.[8] Caglar et al. also showed that administration of probiotic bacterium *Lactobacillus* *reuteri* ATCC 55730, *Bifidobacterium* DN-173 010 or *Bifidobacterium lactis* Bb-12 induced significant reduction of cariogenic S. mutans in saliva.[6,9,10]

However, the role of *Lactobacillus casei* Shirota is not widely studied. In vivo studies of probiotic strains containing *Lactobacillus casei* are lacking. Therefore the aim of this study was to see the effect of probiotic *Lactobacillus casei* Shirota (commercially available as Yakult milk) on the values of mutans streptococci counts in saliva.

MATERIALS AND METHOD

A double blind, randomized, linear crossover study was performed in the Department of Pedodontics and Preventive Dentistry, College of Dental Sciences. Thirty one children residing in Davangere, who had reported for treatment to the Department were selected for the study. All the subjects were explained the need and design of the study and a written informed consent was obtained from parents/legal guardians of all the subjects. The study protocol was approved by the Institutional Ethical Committee of College of Dental Sciences, Davangere.

Probiotic milk (Yakult Danone India Private Limited) and Control milk (Amul-Lite. Mother dairy-GCMMF Limited) were used in the study. Laboratory materials used were Mitis Salivarius Agar Base [Product Code M259-500G, HiMedia Laboratories, India], Potassium Tellurite 1% (1 ml per vial) [Product Code FD052, HiMedia Laboratories, India], Disposable plastic petri-dishes/culture plates (diameter: 90 mm) [Techno MED Plastics, Hyderabad, Andhra Pradesh, India.], Sucrose [Product Number 28105, Fisher scientific, India] and bacitracin zinc salt [Product Code CMS2648, HiMedia Laboratories, India].

Systemically healthy children in the age group of 6-8 years with dmf/DMFT score of ≥ 1 and ≤ 4 were included in the study irrespective of sex.

Consumers of Xylitol products and children with systemic antibiotic, antiseptic mouthwash or probiotic supplements within 8 weeks were not included.

Two sets of similar bottles were marked as A and B by an individual other than the principal investigator which had either probiotic milk (Yakult) or control milk (Amul-Lite - without viable bacteria) in it. The Study period consisted of 57 days including a run in period of 7 days, two intervention periods of 10 days each and a washout period of 30 days. [9] The study had a double-blind, randomized, linear crossover design in which the treated children were then observed for four consecutive time periods. Periods 1 and 3 were run-in and washout periods, in which the subjects were asked to refrain from intake of products containing probiotics, Xylitol, antibiotics and any antiseptic mouthwashes. During periods 2 and 4, the subjects were given 10 ml milk (either A or B) once daily after the lunch for 10 days. The children were instructed to swish the milk for about 2 minutes and then spit as we wanted to examine the local effect of milk and not the systemic effect. Duration of swishing of milk was determined based on the pilot study in which group of children aged between 6-8 years were included and the average time taken by an individual child to drink 60 ml of milk was noted to be 2 minutes. The subjects were encouraged to maintain their normal oral hygiene habits and continue to brush their teeth daily during all four periods.

Microbial evaluation

Samples of non-stimulated whole saliva were collected immediately before and after period 2 and 4. Saliva was expectorated directly into sterile sample bottles. A serial dilution of the collected salivary sample was done in saline. Using spread plate method the diluted salivary sample was streaked on Mitis Salivarius Bacitracin agar (MSB), a selective media for mutans streptococci. The plates were incubated at 37 degree Celsius for 36-48 hours and the number of colonies was counted based on the colony character. The numbers of colonies were counted with the help of digital colony counter (LA660, HiMedia Laboratories, India). These colony counters are lighted surfaces on which the plates are placed, the colonies are marked off with a felt-tipped pen on the outer surface of the plate while the machine keeps counts thus keeping it accurate and consistent.

Statistical method

SPSS, Version 16 software was used to analyze the data. Depending upon the normality of data and since the observations were made in Colony forming units, non-parametric methods were used for analysis. Results of microbiological counts were analyzed statistically by using the Chi - square test for categorical data. Intra group comparisons were made by Wilcoxon signed-rank test. Inter-group comparisons were made by Mann Whitney U test. Z test was used to compare the proportions (% reduction) between two groups.

RESULTS

Results are expressed as mean ± SD and percentage changes. Table 1, represents the intergroup and intra group comparison. Inter-group comparison was statistically highly significant (p=0.001). Table 2 shows the distribution of salivary mutans streptococci in children at baseline and 10 days after the intervention period. Values denote the number of subjects. Table 3 shows that comparison of percentage reduction in children with higher levels (10^5) in two groups. The comparison was statistically highly significant (p=0.001)

DISCUSSION

The key event is that non-pathogenic microorganisms, such as strains of Lactobacilli or Bifidobacteria, can occupy a space in a human biofilm that otherwise would be colonized by a pathogen.[6] Bacteriocins produced by probiotics could function as colonizing peptides, facilitating the introduction and/or dominance of the producer into an already occupied niche.[11] Live microorganisms can inhibit pathogenic microorganism's growth in the oral cavity by producing metabolites such as, organic acids, carbon peroxide and hydrogen peroxide.[5] Ideally, the delivery should be suitable for all ages and especially for very young children, since it has been suggested that exposure to probiotics early in life may facilitate a permanent installation of health promoting strains.[6]

In this study, milk containing probiotic was used. According to Näse L[8] milk and cheese have been shown to be anti-cariogenic in humans by increasing the calcium content in plaque.

To evaluate microbial changes in the present study, saliva samples were collected instead of plaque samples due to less intense microbial count fluctuations in saliva comparing plaque. To avoid inter individual variation effects during saliva stimulation process, unstimulated saliva samples were collected.[12]

Table 1. **Intergroup and Intragroup comparative changes in CFU before and after intervention.**

	CFU Before		CFU After		Difference	% diff	Significance	
	Mean ± SD	Median (Range)	Mean ± SD	Median Range			Z	P
Control group	33.7 ± 41.3	17.2 (5.2-198)	37.6 ± 46.9	19.8 (4.5-194)	-3.9	-7.8	1.20	.23
Probiotic group	53.5 ± 57.9	21.8 (4.8-226)	38.2 ± 43.6	17 (3.2-176)	15.3	21.8	2.98	.003
Control v/s Probiotic	p = 0.12 ns		p = 0.92, ns		p <0.001, s		-	-

A negative sign indicates an increase in CFU.

Table 2. **The distribution of salivary mutans streptococci in children.**

Group	Time	No	mutans streptococci CFU			X^2	P
			$<10^3$ n %	10^{3-5} n %	$>10^5$ n %		
Control group	Baseline 10 days	31 31	5(16.1) 5(16.1)	22 (71.0) 22 (71.0)	4 (12.9) 4 (12.9)	0.00	1.00, ns
Probiotic group	Baseline 10 days	31 31	2(6.5) 3(9.3)	23 (74.2) 24 (77.4)	6(19.4)% 4(3.2)%	0.62	0.73, ns

Values here indicate number of individuals.

In this randomized, double blind linear crossover study, a linear crossover design was chosen so that all subjects received the same treatment and all subjects participate for the same number of periods. Children within age groups of 6 to 8 were included in this study so as to evaluate the effect of *Lactobacillus casei* Shirota on mutans streptococci on newly erupted tooth surfaces as age 6 is the beginning of the second window of infectivity with the eruption of incisors and molars.[13]

In the probiotic group there was a statistically significant (p=0.003) reduction in mutans streptococci score after intervention (Table 1). This would have been due to the fact that *Lactobacillus casei* Shriota affects the oral ecology by specifically preventing the adherence of other bacteria by modifying the protein composition of the salivary pellicle thus preventing the adhesion and affecting the colonization of mutans streptococci.[14]

Our results were in correlation with studies done by Michalek SM[15] in which he concluded that *Lactobacillus casei* strain ATCC 11578 selectively colonized the tongue and saliva and was found in more quantity than *S.mutans* when groups of rats were infected with mixtures of *Lactobacillus casei* strain ATCC 11578 & *S.mutans*. Another study was done by Mortazavi S[16] in which effect of cheese containing *Lactobacillus casei* LAF-TI-L26 was compared with conventional cheese on salivary *Streptococcus mutans*. The authors found a significant reduction of *Streptococcus mutans* count after the intervention period. Haukioja A[14] found that *Lactobacillus casei* Shirota and *Lactobacillus casei* ATCC 11578 diminished the adherence of *S.mutans* when they were allowed to adhere before the streptococci. But when the probiotic strains and *S.mutans* were allowed to adhere simultaneously or the streptococci were allowed to adhere before these probiotic bacteria, these bacteria slightly inhibited the adherence of *S.mutans*.

Michalek[15] compared the virulence of *S.mutans* and *Lactobacillus casei* in gnotobiotic rats and found out that when plaque microflora consisted of 99% *S.mutans,* the caries level was more as compared to combination of 95% *S.mutans* & 5 % *Lactobacillus casei* in plaque. Other studies done to evaluate the effect of different probiotic bacteria also showed significant reduction of *S.mutans* after intervention. [3,6,8,9,15-18]

Amul-lite milk used as a control for the study was 99.7% fat free, skimmed milk. There was slight increase in the colony count but this increase was not statistically significant. This could be attributed to the cariogenicity of milk. Prabhakar AR[19] in their in-vitro study investigated the acidogenicity and cariogenicity of human breast milk and plain and sweetened packaged bovine milk over a period of 12 weeks. The authors concluded that both plain and sweetened packaged bovine milk supported greater bacterial growth and caused more fermentation in an in-vitro caries model making them cariogenic in absence of artificial saliva. Bowen WH[19] did a similar study comparing the cariogenic potential of infant formulae, cow's milk, and a sugar solution and concluded that milk was reasonably cariogenic.

In probiotic group out of 31 children, 2 children had mutans streptococci count below 10^3 before intervention but after intervention, 3 children had a mutans streptococci count below 10^3 and 6 children had mutans streptococci count more than 10^5 before but after intervention, 4 children had a mutans streptococci count above 10^5(p=<0.001). However, there were no changes found with the control group.

In probiotic group greater reduction was seen in number of individuals who had higher levels of mutans streptococci when compared to the individuals who had lower levels of mutans

Table 3. Comparison of percentage reduction in children with higher levels 10^5.

Groups	% Reduction	Z-value	p-value
Control	0%	3.93	<0.001
Probiotic	34%		

streptococci before intervention (Table 2). In our study the comparison of percentage reduction in children with higher levels 10^5 in two groups was 34% in the probiotic group while 0% or no change was seen in the control group (Table 3).

These results were in correlation with studies done on other probiotic strains. Mortazavi S[16] suggested that *Lactobacillus casei* LAFTI L26 was capable of reducing *S.mutans* in subjects with high initial bacterial counts ($\geq 10^5$ CFU/ml). The authors concluded that during intervention, the *S.mutans* count decreased in 46.6% and remained unchanged in 40% and increased in 13.4% of all the subjects. Caglar E[9] found a significant reduction in individuals with $>10^6$ CFU using *Bifidobacterium* DN 173010 in 21 individuals following two week consumption of probiotic yogurt. Caglar E[17] in an in vivo study evaluated the effect of *L. reuteri* ATCC 55730; ATCC PTA 528910 delivered by lozenge in 20 individuals aged 20 years and found significant reduction in individuals with a *S.mutans* levels $>10^6$. Caglar E[6] also investigated the efficacy of ice-cream containing *Bifidobacterium lactis* Bb-12 in 24 individuals aged 20 years on mutans streptococci and found that number of individuals with high mutans streptococci ($>10^5$ CFU) decreased from 56% to zero after intervention. Cildir SK[18] investigated the effect of *Bifidobacterium animalis* subsp. lactis DN-173010 on mutans streptococci in 24 individuals undergoing orthodontic treatment. Subjects consumed 200g probiotic yogurt for 2 weeks. Results showed that number of subjects with high mutans streptococci count decreased from 63 to 21 %.

The study was begun with a presumption that *Lactobacillus casei* Shirota would decrease the counts of salivary mutans streptococci, which was found to be true to a certain extent. Since the sample size was small and study period was short, there is a need for the further long term research on larger samples to validate the obtained data.

CONCLUSION

The following conclusions were drawn from the study:

1. Probiotic milk containing *Lactobacillus casei* Shirota had an inhibitory effect on the mutans streptococci count as compared with control milk.
2. More reduction was seen in the number of individuals who had greater levels of mutans streptococci ($>10^5$) in the probiotic group.
3. Amul-lite milk was found to increase the bacterial growth although not significant.

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