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Comparing the Effectiveness of Different Probiotic Vehicles Containing Fermented Milk on *Streptococcus Mutans*: A Randomized Clinical Trial

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1. Introduction

Probiotics are defined as viable microorganisms that confer health benefits when administered in sufficient doses.¹ These are dietary supplements containing potentially beneficial bacteria or yeasts. They are administered in different quantities that allow for colonization. They help in stimulating health

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promoting flora and also suppressing the pathologic colonization and disease spread.² Most probiotics fall into the group of micro- organisms known as lactic acid producing bacteria and are normally consumed in the form of yoghurt, milk, cheese and other fermented foods. The organisms that have been used as probiotics are primarily certain species of Lactobacilli and Bifidobacteria.³

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Probiotics have been found to be advantageous in combating various diseases such as gastro-intestinal infections, cancer prevention, irritable bowel syndrome, constipation, etc.³ The oral cavity is a port of entry for many microorganisms and can be an important reservoir of enterobacteria. These microorganisms are potential agents in gastrointestinal disorders, hospital infections, and aggravation of oral disease.⁴ The possible mechanisms of action of probiotics in the human body are normalisation of the microbiota, modulation of the immune response and metabolic effects.³

In developing countries such as India, where the major part of the population lacks access to basic health facilities, oral health is a largely neglected area with dental caries widely prevalent across all age groups, more so in children with their fondness for confectionary. Dental caries is a cause of a great deal of continuous discomfort through impaired function and aesthetics and also the costs for operative dental treatment are significant both for individuals and society. Therefore, a need exists to identify individuals at risk for the development of caries, and to target preventive measures and active treatment for these individuals. Spreading the awareness that caries is preventable by adopting simple oral hygiene practices and the use of simple, cost-effective and easily available products is likely to be cost effective.3 An increased knowledge about products that may inhibit the adherence or growth of these bacteria is important and essential. Thus, the objective of the present study was to compare the influence of two probiotic vehicles on mutans streptococci in oral cavity which is primarily responsible for causing dental caries.

2. Methodology

Subjects and study design:

A randomized clinical trial was carried out to investigate the effect of probiotic products on salivary mutans streptococci among 30 volunteered, orally and systemically healthy non-medicating dental students of Pacific Dental College and Hospital, Udaipur, India aged 18 to 22 years (11 males, 19 females). The exclusion criteria were subjects with; habitual consumption of probiotics or xylitol chewing gums, systemic antibiotic medication within 6 weeks, recent repeated topical fluoride treatments, and using oral hygiene aids other than routine teeth brushing. There were two probiotic vehicles namely Choco bar ice cream (net weight of 42g and net volume of 60ml) consisted of bifidobacteria (106/ml) as a probiotic organism and the other one was Yakult (Danone Private Limited, India) containing Lactobacillus casei strain Shirota at 2×10^7 to 10^9 and Bifidobacteria breve at $5 \times$ 107 to 109 CFU/mL

Ethical considerations:

Our research was conducted in full accordance with the World Medical Association Declaration of Helsinki. The study protocol was reviewed and approved by the Institutional Ethical Committee. Written informed consent was obtained from all the study participants after explaining the nature and purpose of research.

Pilot study:

A pilot study was conducted on 10 subjects to assess the feasibility, sample size of the study and to get acquainted to the laboratory procedure of salivary analysis. Based on the results of pilot study, a sample size of 15 participants for each group was obtained using 80% power and significance level of 5%. *Procedure:*

All participants attended three consultations (baseline, after 1 hour and follow up). In the first consultation, all subjects were seen in the morning at 9 a.m., and collection of baseline saliva was performed. Prior to saliva collection, patients were kept seated for 5 minutes, relaxed and silent. After a thorough rinse with water and an initial swallow, unstimulated saliva was collected by allowing the saliva to flow over a period of 5 minutes into a graded test tube by tilting their heads forward. The average flow rate of collected saliva was 0.3 ml/min. Unstimulated saliva was collected from each participant as the analysis of unstimulated saliva is more sensitive than analysis of stimulated saliva.⁵ Moreover, stimulated whole saliva is less suitable for diagnostic applications because the foreign substances used to stimulate saliva tend to modulate salivary content and generally stimulate the water phase of saliva secretion, resulting in a dilution in the concentration of microbial count.6

Following this procedure, using a randomnumber table the participants (n=30) were randomly allocated to Group I (Probiotic Choco bar ice cream) and Group II (Yakult) consisting of 15 subjects in each group by an investigator (Figure 1). Everyone was supplied with their respective product. They were instructed to consume the product and the same procedure of salivary sample collection was repeated after one hour of consumption. None were allowed to eat or drink anything during this period.

Everyone was further instructed to report in the department daily morning at 10 a.m. for consumption of probiotic product. The experimental period comprised of 18 days. They were encouraged to maintain their normal oral hygiene habits and to continue brushing their teeth twice a day during study period. The participants followed these guidelines to improve standardization of data collection. Subsequent to

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JCHR (2023) 13(6), 3685-3690 | ISSN: 2251-6719



baseline and one hour after estimations, each participant underwent the final follow up collection of saliva samples on the 18th day after consuming the product. Neither local nor systemic side effects were noted in the groups.

Microbial evaluation:

Saliva was inoculated on a petridish Mitis salivarius bacitracin agar for mutans streptococci.⁷ The laboratory procedure was meticulously followed with the precaution to prevent contamination of samples using Robertson Meat Broth as transport media. The slides were cultivated at 37° C for 48 hours. The colonies were identified by morphology with the aid of a stereomicroscope with $10 \times$ magnification, and the density of the colony forming units (CFU/ml of saliva) was calculated.

Statistical analysis:

The data obtained were analyzed using SPSS (Statistical Package for Social Sciences) version 11.5 (SPSS Inc., Chicago, Illinois, USA). Non-parametric statistical tests were used as the variables when assessed for normality using the Kolmogorov-Smirnov test showed a non-normal distribution. Comparisons of mean bacterial scores between and within the regimes were performed using Friedman test and Wilcoxon signed rank test respectively. The comparison between test and control group was performed using Mann-Whitney U test. A p-value ≤ 0.05 was considered statistically significant.

3. Results

The mean values of salivary S. mutans between Group I& II and among the Groups were compared at baseline, 1 hr after consumption and follow up are shown in Table I & II. All participants had detectable levels of S. mutans at baseline. In both the groups, the mean value of S. mutans had significantly decreased from baseline to follow up period with a p value of p=0.001 in Group I and p=0.002 in Group II. The mean values of follow up in group I (2.19 X10⁵) was significantly less compared to baseline (8.16X10⁶) and after 1 hr (6.51X10⁶).

The results were similar in Group II also, the mean value of follow up (3.22×10^6) was less significant to baseline (8.7×10^6) and after 1 hr (6.5×10^6) . When the mean ranks of S. mutans were compared and the Wilcoxon signed-rank test was applied within the regimens, a significant difference was found between the 1 hr after and follow up regimens(p<0.05) in both the groups.

4. Discussion

The most common oral disease in developing countries like India is dental caries and periodontitis due to the imbalance in the oral micro flora. Dental caries results from prolonged plaque acidification that leads to the establishment of a cariogenic microflora and demineralization of the tooth. Probiotics has evolved as a new preventive strategy for few oral diseases. The most commonly used organisms in probiotics are Lactobacilli, bifidobacteria, Streptococci, Enterococci and E. coli. Various methods of administration of probiotics have been proposed, but dairy products supplemented with probiotics are natural means of oral administration and easily adopted in dietary regime for adults and children. Dairy products are known to possess caries preventive effects related to a natural high content in calcium and phosphate and the shelf life of ice-cream is 24 months, can be stored for a long time without decrease in number of viable probiotic cells.^{8,9} The present study evaluated the effect of two commercially available probiotic preparations namely Choco bar ice-cream and Yakult consisted of bifidobacterial. The data obtained from the study shows, there were adequate amount of S. mutans at the baseline in both the groups and a drop in S. mutans count was noticed in both the groups after 1hr consumption of probiotic and follow up. The results of the present study showed that the daily consumption of probiotics in both the forms for 18 days reduced the salivary levels of S. mutans counts. Hasslof et al explained in their study, this might be because of the ability of the probiotic bacteria to modify the composition of salivary pellicle by binding and degrading proteins necessary for bacterial adhesion thus preventing adhesion of S. mutans to oral biofilm.¹⁰ Twetman et al carried out a study to assess the effectivity of probiotics in caries reduction in children and reported a significant caries reduction in 3 to 4 year-old children after 7 months of daily consumption of probiotic milk.11 Keller et al12 concluded that selected lactobacilli displayed coaggregation activity and inhibited growth of clinical mutans streptococci. The growth inhibition was strainspecific and dependent on pH and cell concentration. B. adolescentis SPM1005 cells decreased the growth of S. mutans, which is a risk factor for dental caries. Therefore, authors suggested that this Bifidobacterium strain may be a useful probiotic microorganism for prevention of dental caries that does not have adverse effects.

While comparing the efficiency of the vehicle of probiotics (ice cream and drink), there was significant reduction in S. mutans level in subjects who consumed ice cream (Group I) after follow up. This signifies the caries inhibiting effect of ice-cream with bifidobacteria

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JCHR (2023) 13(6), 3685-3690 | ISSN: 2251-6719



despite the sugar content of the ice-cream The mechanism of action of oral probiotics is not clearly understood, but several local and systemic effects were described in literature, including adhesion, co-aggregation, competitive inhibition, production of organic acids and bacteriocin-like compounds and immune-modulation and probiotics acts as a biofilm and occupies the space which could be occupied by pathogens in the absence of the film and keeps the pathogen away. Probiotic effects of ice cream would be more as compared to Yakult drink because probiotic bacteria adhere to the oral mucosa and dental tissues become the part of the biofilm to compete the growth of S. mutans.

Teughels W¹³ and Singh et al¹⁴ reported that probiotic ice-cream containing Bifidobacterium lactis Bb-12 ATCC27536 and Lactobacillus acidophilus La-5 can reduce the levels of certain caries-associated microorganisms in saliva. Kruger et al¹⁵ performed a double blind, randomized crossover study to evaluate the effect of Bifidobacteria. A statistically significant reduction of salivary mutans streptococci was recorded after the probiotic yoghurt consumption containing Bifidobacterium, which was in contrast to the controls.

Unstimulated saliva was collected from all the subjects for the analysis. Kaufman and Lamster had documented that analysis of unstimulated saliva is more sensitive than stimulated saliva.⁵ According to Miller et al stimulated saliva is less suitable for analysis since some stimulants can modulate salivary content and watery saliva results in dilution in concentration of microbial count. ¹⁶

The present study assessed only the short-term effect of Lactobacilli and bifidobacteria and demonstrated significant reduction in cariogenic S. mutans count in saliva. Caglar et al recommended constant uptake of probiotics for its permanent colonization, so a continuous regular, almost daily intake is needed.¹⁷ Further long-term studies are required to evaluate the permanent colonization.

5. Conclusion

The study had shown decreased number of S. mutans in subjects who consumed probiotic Choco bar ice cream during follow up in saliva. Unfortunately, the study groups were relatively small and fairly short.

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JCHR (2023) 13(6), 3685-3690 | ISSN: 2251-6719

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Table 1.	Comparison	of mean streptococcus	mutans and lactobacilli	count between test and	l control groups
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Groups	Time of	Streptococcus Mutans		
(11-50)	assessment	Mean ± SD	F value	P value
Group I	Baseline	$8.1648 \times 10^6 \pm 1.5240 \times 10^6$	22 6279	0.001*
(n=15)	After 1 hr	$6.5127{\times}10^6{\pm}1.9890{\times}10^6$	22.0279	0.001
	Follow up	$2.19{\times}10^5 {\pm}7.1572{\times}10^{5{\ast}{\ast}}$		
Group II	Baseline	$8.7092 \times 10^6 \pm 1.589 \times 10^6$	28 4315 0 002	0.002*
(n=15)	After 1 hr	$6.9144{\times}10^{6}{\pm}1.2582{\times}10^{6}$	20.4313	0.002
	Follow up	$3.2253 \times 10^{6} \pm 5.3908 \times 10^{6**}$		

Test applied: Friedman test (between regimes), *p<0.05 statistically significant

www.jchr.org

JCHR (2023) 13(6), 3685-3690 | ISSN: 2251-6719



Test applied: Mann-Whitney U test (between test and control group), **p<0.05 statistically significant

Table 2. Comparison of mean rank of streptococcus mutans and lactobacillus count within regimes among test and control groups.

Crowna		Streptococcus Mutans			
(n=30)	Time of assessment	Mean Positive Rank	Mean Negative Rank	P value	
Cuoup I	Baseline & 1 hr	8.25	7.91	0.0730	
(n-15)	1 hr & Follow up	0.00	8.00	0.0003*	
(II -13)	Baseline & Follow up	2.00	8.43	0.0002^{*}	
Crown II	Baseline & 1 hr	8.33	7.78	0.570	
(n-15)	1 hr & Follow up	6.33	8.42	0.020^{*}	
(II -13)	Baseline & Follow up	8.00	8.00	0.003*	

Test applied: Wilcoxon signed rank test (within regimes), *p<0.05 statistically significant.