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Effect of Short-term Consumption of Amul Probiotic Yogurt Containing *Lactobacillus acidophilus* La5 and *Bifidobacterium* Lactis Bb12 on Salivary *Streptococcus mutans* Count in High Caries Risk Individuals

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Abstract

Aim: This study aims to study the effect of short-term consumption of probiotic yogurt containing *Lactobacillus acidophilus* La5 and *Bifidobacterium* lactis Bb12 on salivary *Streptococcus mutans* count in high caries risk individuals. **Materials and Methods:** A double-blind randomized control trial was conducted, and 70 high caries risk individuals with a salivary *S. mutans* count of more than 10^6 CFU/ml of saliva were followed for 4 weeks. Participants ingested 100 g yogurt containing *L. acidophilus* La5 and *Bifidobacterium* lactis Bb12 or yogurt without the two probiotic bacteria once daily at the end of meals for initial 2 weeks. Salivary *S. mutans* were enumerated in the laboratory by selective culture media. **Results:** A statistically significant reduction ($P < 0.05$) of salivary *S. mutans* was recorded after probiotic yogurt consumption with minimal residual effect, which was in contrast to the controls. **Conclusion:** *L. acidophilus* La5 and *Bifidobacterium* lactis Bb12 present in the yogurt were effective in reducing the *S. mutans* levels in saliva.

Keywords: *Bifidobacteria, caries prevention, lactobacilli, probiotics, Streptococcus mutans*

Introduction

Worldwide dental caries has affected 60%–90% of school children and nearly 100% of adults.[1] It is the most common and chronic microbial disease of complex etiology predominantly causing tooth loss. Reduction in dental caries is one of the objectives to achieve the Global Goals 2020. However, it has shown increasing trend in the developing countries including India.[2]

Research in the field of caries prevention has been focusing on ways for reducing or totally eradicating cariogenic flora from the oral cavity. However, most of the studies have shown that it is difficult to completely eliminate *Streptococcus mutans* from oral cavity by mechanical and chemical control. In an era where increasing health-care costs, rising food literacy, and the Hippocratic concept of "let food be thy medicine and medicine be thy food" has led to discovery of "functional foods" that have moved into the corporate mainstream,[3] probiotics with their wide range of applicability have been believed to confer various beneficial health effects. Theoretically, oral microbial ecological alteration with these bacteria and a long-term reduction of *S. mutans* by these bacteria could imply to a reduced risk of initial lesions and also would clarify whether this approach could be an alternative strategy for the reduction of pathogenic *S. mutans* count[4] and thus an alternative to caries control strategy.

Lactobacillus acidophilus La5 and *Bifidobacterium* lactis Bb12 are widely available in Indian markets with their focus on general health, oral ingestion being the common route of administration. We hypothesized that a short-term consumption of probiotic yogurt containing *L. acidophilus* La5 and *Bifidobacterium* lactis Bb12 might reduce salivary *S. mutans* count in high caries risk individuals. Being an intermediate endpoint, the study was undertaken to compare the salivary *S. mutans* levels before and after the short-term consumption of above-mentioned probiotic bacteria.

Materials and Methods

Prestudy procedure

Ethical approval was obtained from the Ethics committee, Sumandeep Vidyapeeth,

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Piparia, Vadodara. The sample size was calculated to be 58 (29 control and 29 intervention) based on the previous study[4] but was increased to 70 (35 control and 35 intervention) to tackle any expected attrition during the study. The confidence interval was taken to be 95% and power was set at 80%.

Training, calibration, and personnel

The field procedure for identifying the initial carious lesions using Nyvads criteria[5] was carried out by a single trained calibrated (Kappa coefficient = 0.89) investigator (Principal investigator) using the American Dental Association type III criteria,[6] while the laboratory investigations were carried out by an adequately trained and experienced microbiologist.

Selection of study subjects

A total of 105 individuals with informed and written consent were invited on a convenience basis for the study, of which those having at least one active carious lesion were considered for microbiological tests.[7] Further after the microbiological test 70 individuals with a salivary *S. mutans* count of more than 10^6 CFU/ml of saliva (microbiological selection criteria) were considered to have high caries risk[8] and were included in the study.

Exclusion criteria

Individuals with Habitual consumption of xylitol products or those on systemic antibiotic medication or fluoride treatment or those consuming any other probiotic product within the past 6 weeks or during the study were excluded from the study.

Preparation of mitis salivarius bacitracin agar – a selective culture media

The mitis salivarius agar powder recommended for Streptococci and bacitracin were obtained from HiMedia Laboratories Limited, Mumbai. The process of making culture media was carried out as per the instructions provided by the manufacturer while addition of bacitracin was done as recommended by Gold *et al*., 1973.[9] One ml of sterile 1% potassium tellurite solution was also added to it for staining the colonies. The pH of the agar was maintained at 7.4 at 25°C.

Study procedure

A double-blind randomized control trial was conducted. Randomization of individuals was done by lottery method and commercially available 100 g of commercially available Amul Masti probiotic yogurt containing *L. acidophilus* La5 and *Bifidobacterium* lactis Bb12 in the quantity of 10^6 CFU/100 g of yogurt was given to the experimental group while 100 g of Amul Masti yogurt without probiotic bacteria was given to the control group, once daily at the end of meals (so as to prevent the destruction of bacteria from gastric acids[10]), for 2 weeks. Individuals were asked to refrain from xylitol products, antibiotic medication, fluoride containing products, or any other probiotic product during the 4-week study.

Saliva sample collection and culture process

Paraffin-stimulated saliva samples were collected at baseline, immediately at the end of the intervention period (2 weeks) and at the end of the 4th week between 9.00 a.m. and 11 a.m. to minimize the effects of diurnal variation. The participants were asked to refrain from eating and drinking at least 90 min before saliva collection and to avoid swallowing during collection. The collected samples were then transferred to the laboratory immediately and cultured on selective media after serial dilutions. After 48 h of the incubation period, *S. mutans* appeared on the culture plate as blue-gray small, rough, raised, and adherent colonies. Colonies so identified were counted using a standardized digital colony counter. The number of colonies observed was then multiplied by the dilution factor of 10^5 to obtain the colony forming units of bacteria per ml of saliva.

Statistical analysis

ANOVA with repeated measures was performed to compare the intragroup *S. mutans* count respectively at different follow-ups. The intergroup comparison was done at baseline, at the end of 2 weeks and at the end of the 4th week using unpaired *t*-test.

Results

Table 1 illustrates the intergroup comparison of mean number of *S. mutans* count in case and control group at baseline, 2 weeks and 4 weeks using unpaired *t*-test. The count showed a nonsignificant difference at baseline and at the end of 4 weeks period but was significantly different at the end of 2 weeks' intervention period.

Table 2 shows the results for intragroup comparison using repeated measure ANOVA.

Mauchly's test of sphericity for the case group showed that there were significant differences between the "variance of differences" of the *S. mutans* count at baseline, 2 weeks and 4 weeks as a result the readings were subjected to Hyunh and Feldt's test to compare difference in *S. mutans*

Table 1: Intergroup comparison of mean *Streptococcus mutans* counts in case and control using unpaired *t*-test

Time	Group	*n*	Mean ($\times 10^7$)±SD	SEM	*P*
Baseline	Control	35	1.3229±0.06819	0.01153	0.53
	Case	35	1.3326±0.06349	0.01073	
2 weeks	Control	35	1.3163±0.06495	0.01098	0.00*
	Case	35	1.1711±0.05138	0.00868	
4 weeks	Control	35	1.3197±0.06474	0.01094	0.84
	Case	35	1.3169±0.06296	0.01064	

*Significant. SD: Standard deviation; SEM: Standard error of mean

Table 2: Repeated measure ANOVA for intragroup comparison

Variable	Group	Mauchly's W	*P*	Huynh-feldt		Wilk's lambda		Pair-wise comparison	*P*
				F	*P*	*F*	*P*		
Streptococcus mutans count	Case group	0.748	0.008*	169.60	0.00*	NA	NA	Baseline versus at the end of 2 weeks (1st follow-up)	0.000
								At end of 2 weeks (1st follow-up) versus at end of 4 weeks (2nd follow-up)	0.000
								Baseline versus at end of 4 weeks (2nd follow-up)	0.534
	Control group	0.977	0.683	NA	NA	2.826	0.074*	NA	NA

*Significant. NA: Not applicable

count during the 4-week period which was of highly significant value, F (1.664, 34.000) = 169.607, *P* = 0.000. Thus, a pairwise comparison was done further that showed a significant difference of *S. mutans* between the baseline and 1st follow-up and also 1st and 2nd follow-up with a *P* value of 0.000. The difference of count was not significant between baseline and 2nd follow-up.

Mauchly's Test of Sphericity for the control group showed that there were no significant differences between the "variance of differences" of the *S. mutans* count at baseline, 2 weeks and 4 weeks in control group. As a result, the readings were subjected to Wilks Lambda test which suggested that there was no significant difference of the *S. mutans* count during the 4 weeks period. Thus, a pair-wise comparison was not done in control group.

Table 3 shows the effect of time and interaction between time and intervention on *S. mutans* count in all the study participants. The effect was observed using Hyunh and Feldt's correction and a significant difference was observed in the *S. mutans* count as the time passed, *F* (1.663, 113.117) = 164.780, *P* = 0.000 and also there was a significant effect of interaction between the time and intervention on *S. mutans* count, *F* (1.663, 113.117) = 168.899, *P* = 0.000.

Discussion

Antibiotic resistance, with the emergence of multiple resistant strains, is an increasingly important global problem. Oral infections constitute to some of the most common and costly forms of infections in humans.[11] It is a well-established fact that caries is characterized by colonization of the tooth surface biofilm (dental plaque) by mutans streptococci–in humans, while *S. mutans* have been implicated as specific organism associated with initiation of caries.[12] The ability of these microbes to adhere firmly to the surfaces of salivary protein-coated teeth and plaque biofilm and increase in numbers by both growth and recruitment from the planktonic phase (salivary suspension) by auto-aggregation on exposure to dietary sucrose is an important event in caries formation and progression.[13]

The concept of microbial ecological change with probiotic bacteria as a mechanism for preventing dental disease is therefore important for prevention of dental disease.[11]

Randomized controlled trial is the most rigorous way of determining whether a cause-effect relation exists between treatment and outcome and also rules out the selection bias and therefore it was used in the present study. Blinding at the level of participants and laboratory investigator helped to rule out the ascertainment bias. The present study aimed at evaluating the combined effect of probiotic bacteria *L. acidophilus* La5 and *Bifidobacterium* lactis Bb12 on salivary *S. mutans* count with yogurt as a vehicle in high caries risk individuals. The effect of probiotic bacteria was evaluated without exercising control on individuals routine oral hygiene procedures and dietary habits (except for control over intake of other probiotic products) so their effect in real-life situation could be observed.

In our study, a significant difference in *S. mutans* count was seen in the case group during the 2-week period suggesting that the probiotic bacteria *L. acidophilus* La5 and *Bifidobacterium* lactis Bb12 were effective in reducing the *S. mutans* count in saliva and for this the null hypothesis was rejected. These results were similar to that of Singh *et al*., Caglar *et al*., Cildir *et al*., Chinnappa *et al*., Srivastava *et al*. and Yousuf *et al*.[14-19]

The decline in the *S. mutans* count observed in the present study can be attributed to the fact that probiotics exert health benefits on the consumers. The various mechanisms of action of probiotics in the oral cavity are listed in Table 4.

The probiotic bacteria, *Bifidobacterium*, have been found to be quite acidogenic in the oral cavity while lactobacillus is the most acidogenic among other lactic acid producing bacteria. The duo are also heterofermentative in nature, i.e., they produce both lactic acid and acetic acid. Bifidobacteria also have the ability to bind to *Fusobacterium nucleatum*-covered hydroxyapatite and lactobacilli have been shown to temporarily colonize the oral cavity.[20] These properties of both *Bifidobacterium*

Table 3: Effect of time and combined effect of time and intervention on *Streptococcus mutans* count

Variable	Test	*F*	*P*
Time	Huynh-feldt	168.899	0.000*
Time × intervention	Huynh-feldt	164.780	0.000*

*Significant

Table 4: Mechanism of action of probiotics

The production of antimicrobial substances by the bacteria like
Organic acids, hydrogen peroxide, bacteriocins
Binding of the bacteria to sites in oral cavity
To compete with pathogens for adhesion sites by modifying the composition of salivary pellicle
Involvement in metabolism of substrates thus competing with oral microorganisms for substrates available
Immuno modulatory action of bacteria
Stimulating nonspecific immunity
Modulating humoral and cellular immune response
Modification of oral conditions by bacteria
By modulating pH
Modifying the oxidation reduction potential

and lactobacillus render them the ability to adhere to the oral mucosa and dental tissues as part of the biofilm and compete with the growth of dental pathogens to be effective against oral infections and could be responsible for the observed decline in *S. mutans* count in saliva in the current study.

A significant increase in the *S. mutans* count was observed at the end of fourth from that at the 2nd week, and this could be due to the minimal residual effect of the intervention after its discontinuation. The difference in *S. mutans* count in the control group during the 4-week period was found to be nonsignificant suggesting that the normal curd without probiotic bacteria was ineffective in reducing the *S. mutans* count in saliva. These results were in accordance with Mahantesha *et al*. and Sutula *et al*. observed a significant reduction of *S. mutans* level after the study period who also showed that there was no significant reduction in *S. mutans* count when compared to the baseline data or levels after the washout period.[21,22]

Probiotics incorporated into dairy products neutralize acidic conditions in the mouth and interfere with cariogenic bacteria.[23] Ferrazzano *et al*. suggested that the vehicle for administration of probiotics should be of milk origin due to contained casein phosphopeptides that have an inhibitory effect on demineralization and promote the remineralization of dental enamel.[24] Fermented dairy products are considered to be excellent vehicle for delivering probiotics because of a synergistic relationship between components in dairy products and probiotic cultures.[25] Hence, Yogurt was used as a vehicle in the present study.

The present study group comprised of young adults with the age of 18 years and above to rule out the inclusion of legally incompetent subjects (such as a [minor] child) in research on the effect of probiotics on oral health. The individuals selected for the microbiological test during sample selection were only those having initial active carious lesions, as acidogenic potential of the probiotic bacteria could have played a role in deep dentine caries progression.

In the current study, the sample size was determined by fixing the power of the study at 80% hence the study could not have failed to show the difference in outcomes in case it existed and therefore these results could be interpreted as positive results.

Conclusion

It was concluded from the present study that the consumption of probiotic bacteria *L. acidophilus* La5 and *Bifidobacterium* lactis Bb12 was effective in the reduction of salivary *S. mutans* but showed a minimal residual effect, and the approach could thus act as an alternative strategy for the reduction of pathogenic *S. mutans* count in oral cavity.

Limitations

The short intervention time in the present study was a limitation.

Recommendations

1. Individual effects of *Bifidobacterium* lactis Bb12 and *L. acidophilus* La5 on *S. mutans* and tooth structure need to be evaluated
2. The compatibility of mode of delivery, i.e., vehicle also needs to be assessed for the two bacteria separately as the two bacteria individually might interact differently with the vehicle
3. The encouraging findings from the present study also call for an intervention study in caries-prone children.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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