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Evaluation of Immunostimulatory Effect of Probiotic Lactobacillus Sporogenes In Oral Environment By Quantitative Analysis of Salivary IgA Levels In Children With High Caries Risk

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Abstract

Background: Probiotics are live micro-organisms which have an immunomodulatory effect by confering health benefits to the host when administered in adequate amounts. They enhance the production of salivary IgA (SIgA) which is an indicator of mucosal immunity by making the environment less conducive for cariogenic pathogens. This study aims to evaluate the SIgA levels after **consumption** of probiotics at different time intervals.

Methods: Forty two children aged 6 to 12 years were randomly divided into the study (Group1) and control (Group 2) group. Group1 received milk with Lactobacillus sporogenes and Group 2 received plain milk for 30 days. Unstimulated saliva samples were collected at baseline(T0), 30 days after probiotic intervention(T1) and three months post intervention(T2) and evaluated quantitatively for increase in SIgA levels using ELISA immuno-enzymatic colorimetric method.

Results: The results showed an increase in SIgA levels from T0 to T1 for Group 1 (probiotic) while it decreased among Group 2 (non probiotic) subjects. This difference over this time interval among probiotic and non-probiotic group was found to be **statistically significant** suggesting an increase in the levels of SIgA over the period of 30 days (p=0.05). There was no significant difference seen when intra and inter-group comparison was made at other time intervals.

Conclusion: Probiotics (L.sporogenes) increases SIgA levels as long as the supplementation is done however no long term effect is seen. It can temporarily enhance SIgA levels during the intervention period thereby decreasing further caries progression by its immuno-protective and immuno-stimulatory role against dental caries.

Introduction

Probiotics have been studied for systemic benefits over years and offlately an increased interest is developing on their oral benefits, their immune stimulating and caries preventive roles. [1].WHO has defined probiotics as “living microorganisms that confer a health benefit for the host when administered in sufficient amounts” and the term

probiotic was initially proposed by Lilley and Stillwell in 1965.[2] Lactobacillus acidophilus was first probiotic to be introduced by Hull et al in 1984 followed by Holcombh et al in 1991.[3]

The mechanism of action of probiotics is still not clear. However they have shown an immuno stimulatory effect by modulating the inflammatory response, modifying the environment and competing with the binding sites and nutrients of pathogens.[3] They also influence level of inflammatory mediators in crevicular fluid.[4]

They have shown to reduce streptococcus mutans counts after short term administration and thereby showing their oral benefits. Many probiotic bacteria and their strains have been explored, but the most widely used belong to the genera Lactobacillus and Bifidobacterium. Lactobacillus rhamnosus GG ATCC 53103 (LGG) which was named after discoverers Gorbach and Goldin on **17th April 1985** is one of the most extensively researched probiotic strain having a potential inhibitory activity against cariogenic streptococcus species.

Intake of probiotic lactobacilli enhances the production of salivary IgA (SIgA) which has been widely used as an indicator of mucosal immunity. SIgA has an immunological control over dental caries by preventing the adherence of cariogenic microorganisms to hard surfaces and inhibiting the activity of glycosyltransferases.[5] Higher titres of SIgA levels are seen in people with low dental caries.[6]

The aim of this study was to evaluate the levels of salivary IgA concentration in children who consumed milk enriched with probiotic Bifilac sachet (Lactobacillus sporogenes) daily for a period of 30 days against true placebo group consuming plain milk.The levels were measured at baseline (T0), after 30 days(T1) and three months(T2) post supplementation.

Methodology

Source of Data and Sample Collection

The present study was conducted at a primary government school, Greater Noida and ITS Dental College Hospital and Research Centre, Greater Noida where 42 children aged between 6 to 12 years were included in the study fulfilling the criteria of DMFT/dmft score ≥3 and non habitual use of probiotics. Children with any history of systemic diseases or respiratory illness , recent intake of antibiotics or topical fluoride treatment in past 3 months , periodontal treatment 6 months prior , athletes due to decrease in IgA levels [7] , intake of sulfasalazine drug , allergy to dairy products and habitual use of dairy probiotics or xylitol chewing gums were excluded from the study. An ethical clearance was obtained from the ethical committee ITS Dental College, Greater Noida Ref.No. Director –PG Studies/ ITSCDSR/1./2018/138 and a proper consent was taken from the principal of the school and parents of the selected children. A flowchart of the study is outlined in **Figure 1**. All children were asked to immediately report if any adverse side effects were noted. Compliance was checked by the principal overseeing the children and dentist by maintaining an attendance register.

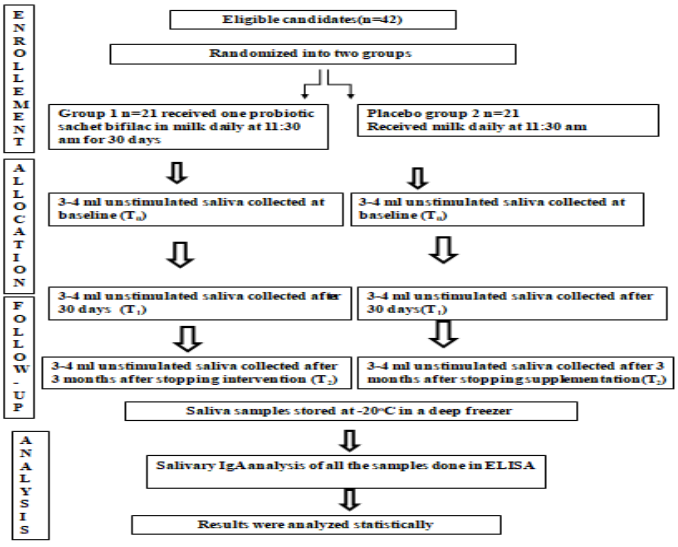


Figure 1: Flow chart of participant

Study Design

A single blind placebo controlled trial was planned where in children were randomly divided into test (probiotic) and control (non-probiotic) group after selecting coloured balls. The test group 1 (n = 21) received a sachet of probiotic Bifilac with 200 ml milk at 11:30 am daily and the control group 2 (n=21) received only 200 ml milk at same time for a period of 30 days.

Collection of Saliva Sample

At baseline(T0), 30 days after completion of intervention (T1) and 3 month after discontinuation of supplementation (T2), 3-4 ml of unstimulated saliva sample was collected in a vial (without any oral movements) from each participant of both groups. Children were asked to pool the saliva in the floor of the oral cavity and to spit intermittently. The child was asked not to eat or drink (except for water) one hour before saliva collection to minimize possible food debris and stimulation of saliva. Each salivary sample was collected in precoded collecting vials, transported in icebox and stored in the research laboratory in a deep freezer at -20 ℃. The procedure was done in research laboratory at the ITS Dental college.

Coding Of Vials

The vials were coded in 3 segments where alphabet A and B were used to denote test group and control group respectively. Serial number 1 to 21 designated to each individual child where third value denotes the time at which sample was collected where 0 represents T0, 1 represents T1 and 3 represents T2 e.g. A01.0 depicts the first child in probiotic group representing baseline saliva sample .

Laboratory Experiment

All the stored salivary samples were brought to room temperature ,thawed, centrifuged at 3000 rpm for 10 minutes and supernatant was collected. The saliva was diluted in the eppendorf tubes with the solutions in the ELISA kit Salivary IgA ELISA(DRG Lot N 4139C-2) **(Figure 2)** as per the manufacturer`s instruction manual. The samples were then pipetted into microtitre wells .

Figure 2

After instructed incubation time the bound/free separation is performed by a simple solid-phase washing followed by the reaction of enzyme in the bound- fraction with the substrate (H_2O_2) and the TMB Substrate developing a blue colour that changes yellow**(Figure 3)** after 15 minutes when the Stop Solution (H_2SO_4) is added.

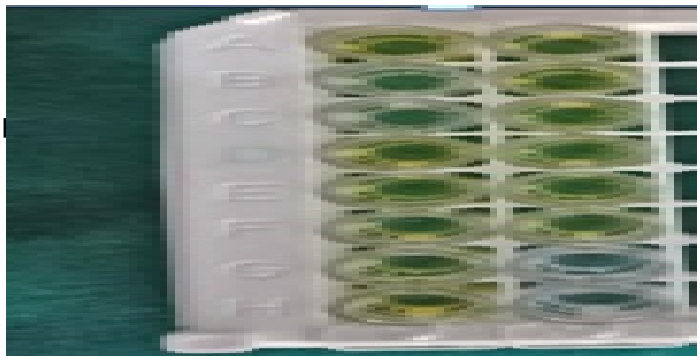


Figure 3

The color intensity is proportional to the SIgA concentration in the saliva sample.The change in colour was measured in the ELISA machine where quantitative determination of IgA at three different point of time in human saliva was done by calculating optical density at dual wavelength [450nm and 620 nm]. This method allows the determination of IgA from 0.5microgram/ml to 400microgram/ml.

Stastical Analysis

Concentration of all the samples was calculated using a linear regression formula. The data collected was analysed using Statistical Package for Social Sciences (SPSS version 21.0) software and the differences were considered significant when the p-values were less than equal to 0.05.

Results

A total of 42 children (age ranges 6-12 years, n =21 for males and n =21 for females) completed this 4 month study. No side effects from probiotic or consumption of milk was reported.

Before conducting the experiment standardization and calibration of ELISA kit was done. The standard measurement results are represented by a linear curve which represents the relationship between calculated optical density of calibrated samples and their known concentration as shown in **graph 1**.

The IgA concentration of the unknown saliva samples were then calculated through a standard curve using linear equation formula y= 0.0045x+0.1891 R^2 =0.9315. This was known as absorbance value

Here x axis represents the concentration and y axis represents the optical density

Graph 1.

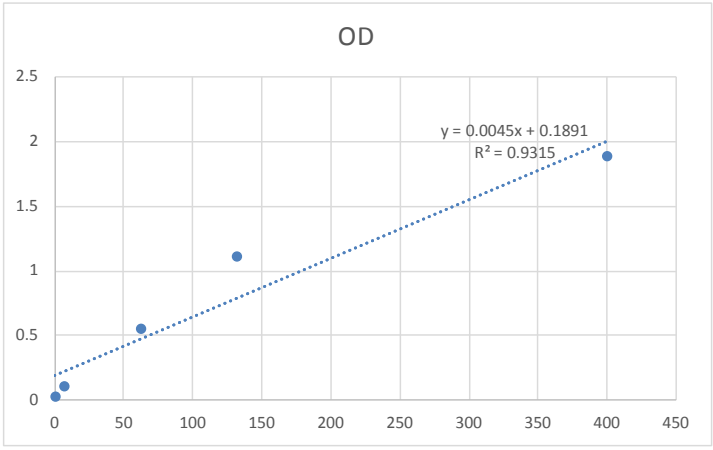
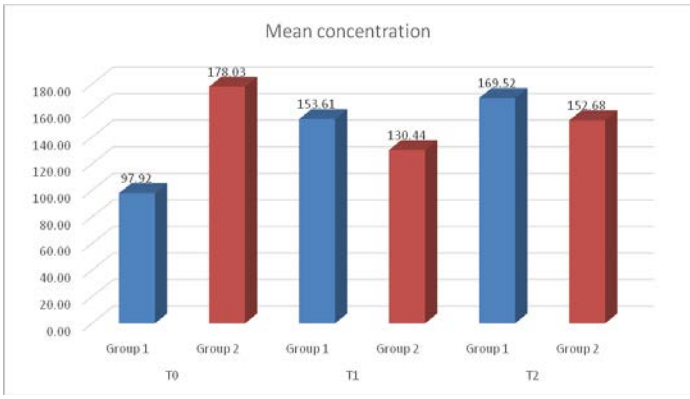


Table 1: Intergroup comparison of mean concentration of SIgA at different points of time.

Concentration at	Gr	N	Mean	Std. Deviation	P[c] value
T0	1.00	21	97.9164	103.72394	0.521, NS
	2.00	21	178.0317	246.20649	
T1	1.00	21	153.6074	113.20434	0.297, NS
	2.00	19	130.4421	141.96081	
T2	1.00	20	169.5189	157.92708	0.299, NS
	2.00	19	152.6807	186.04088	

[c]Mann Whitney U test, NS- Not significant



Graph 2

Table 1 shows at T0 mean concentration levels of SIgA was found to be lower among Group 1 subjects as compared to Group 2 subjects, but this difference failed to reach the level of statistical significance.

At T1 mean concentration levels of SIgA was found to be higher among Group 1 subjects as compared to Group 2 subjects, however this difference failed to reach the level of statistical significance.

At T2 mean concentration levels of SIgA was found to be higher among Group 1 subjects as compared to Group 2

subjects, but this difference failed to reach the level of statistical significance.

Table 2: Intragroup comparison of mean concentration of SIgA between different points of time among Group 1 (Probiotic group) subjects

Concentration at	gr	N	Mean	Std. Deviation	P[d] value	Intra group pairwise comparison
T0	1.00	21	97.9164	103.72394	0.212, NS	T0 & T1-0.054, NS
T1	1.00	21	153.6074	113.20434		T1 & T2- 0.940, NS
T2	1.00	20	169.5189	157.92708		T0 & T2- 0.126, NS

[d]Kruskal Wallis test, NS- Not significant

Table 2 showed that the concentration increased from T0 to T1 and from T1 to T2, but this increase was not statistically significant.

Table 3: Intragroup comparison of mean concentration of SIgA between different points of time among Group 2 (Control group) subjects

Concentration at	Group	N	Mean	Std. Deviation	P[d] value	Intra group pairwise comparison
T0	2.00	21	178.0317	246.2064	0.801, NS	T0 & T1-0.398, NS
T1	2.00	19	130.4421	141.9608		T1 & T2- 0.811, NS
T2	2.00	19	152.6807	186.0408		T0 & T2- 0.355, NS

[d]Kruskal Wallis test, NS- Not significant

Table 3 showed that the concentration decreased from T0 to T1 and then increased from T1 to T2, but these differences were not statistically significant.

Table 4: Intergroup comparison of change in mean concentration of SIgA between different points of time.

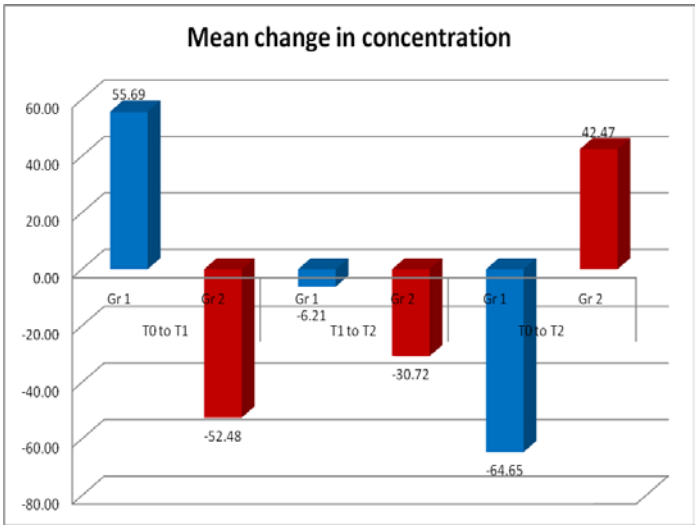
Change in concentration change from	Group	N	Mean	Std. Deviation	P[c] value
T0 to T1	1.00	21	55.6910	141.03681	**0.05, S**
	2.00	19	-52.4772	212.13093	
T1 to T2	1.00	20	-6.2133	191.47740	0.792, NS
	2.00	18	-30.7198	168.86696	
T0 to T2	1.00	20	-64.6511	172.34253	0.081, NS
	2.00	19	42.4690	203.13986	

[c]Mann Whitney U test, NS- Not significant

Table 4 and graph 3 showed that from time interval T0 to T1, mean concentration among group 1 subjects increased while it decreased among Group 2 subjects. This difference among probiotic and non-probiotic group was found to be **statistically significant** suggesting an increase in the levels of SIgA over the period of one month.

From T1 to T2, mean concentration decreased among both group children was not statistically significant.

From T0 to T2, mean concentration among group 1 subjects decreased while it increased among Group 2 subjects. But this difference was not found to be statistically significant suggesting probiotics don't have a long lasting effect and needs to be replenished at regular intervals.



Graph 3

Discussion

Probiosis/**bacteriotherapy** or bacterial replacement therapy refers to a method of combatting infections by the administration of non pathogenic bacteria to displace pathogenic microorganisms from the human body for host benefit.[8]

Probiotics have shown to improve the body's immune system through its ability to induce SIgA formation, macrophage activation, modulation of pro-inflammatory cytokines, production of antioxidants like vitamins and lactic acid. They have shown a role in stimulating local immunity by producing bacteriocins against pathogenic bacteria causing dental caries thus modifying the oral environment and competing with the pathogens for nutrients at the binding sites.[3,9]

Nase et al reported significant reduction in dental caries after supplementation with L.rhamnosus for 7 months.[10]

Holgerson et al reported presence of LB in breast milk inhibited the growth of MS which may affect caries development in children.[11] Preventive role of probiotic L.rhamnosus GG in milk for 7 months in 3 to 4 year preschool children showed a 75% reduction in caries prevelance.[12]

Pahumunto et al reported consumption of milk powder containing L. paracasei SD1 resulted in a reduction of both salivary MS and delayed new caries development, and the strain is safe for use in young children.[13] **Kotani et al** stated that oral intake of L.pentosus strain b240 for 12 weeks significantly accelerated SIgA secretion showing intake of this strain increases SIgA secretion via activation of the gut mucosal system.[6]

Secretory IgA is the first line of defense produced by local plasma cells (PCs) and main immunoglobulin in secretions which invade the mucosal surfaces.[14] They boost oral immunity by preventing microbial adherence, neutralizing enzymes, toxins, and viruses; or by acting in synergy with other factors such as lysozyme and lactoferrin, blocking the epithelial receptors and inhibiting the attachment of pathogenic bacteria, especially Streptococcus mutans to epithelial cells.[15]

Immune modulatory effects of probiotic in general and even for the Lactobacillus genus are strain specific and its has been reported that not all probiotics possess the ability to confer beneficial effects to the oral health.[16] **Ericson et**

al used chewing gum and found no significant impact of bacteria on the SIgA concentration but were able to show a post treatment increase in total IgA% per protein after intake of L.reuteri.[1]

The clinical studies on immune regulating probiotics are inconclusive .**Wattanarat et al.** found increased levels of HNP (Human neutrophil peptides)[17] and SIgA levels in saliva of children after probiotic administration. Conflicting results of lower SIgA levels with high caries were also seen in cigarette smokers and in children with chronic stress, athletes, loneliness and depression.[7]

In our study a sample of 42 children were selected and divided into two groups to study the possible immune modulating properties of oral probiotic supplements of L.sporogenes on healthy children with high risk of dental caries .The experiment group received probiotic in milk and control group received plain milk for 30 days. Saliva samples were collected from all the subjects at baseline(T0), after 30 days (T1) and three months post intervention (T2) .The collected samples were stored in a deep freezer at -20^{0}c. Quantitative determination of Salivary IgA levels was done using indirect sandwich ELISA test which is immunoenzymatic colorimetric method .

In our study the oral intake of polybacterial immunomodulator BIFILAC was done which contained killed Lactobacillus sporogenes a gram-positive, spore-forming, lactic acid producing bacteria . It is obtained from fermentation of Vitamin C (ascorbyl palmitate) which is derived from corn dextrose fermentation and palm oil. It has been seen to induce a strain – specific IgA response in the saliva of healthy children with high dmft/DMFT score.

Our study showed significant difference over time interval T0 to T1 amongst probiotic and non probiotic group to be statistically significant showing as long as the probiotic supplementation of L.sporogenes was done an immunostimulatory effect of increased SIgA concentration was seen. However, long term benefits of probiotic wasn't seen from baseline to the follow up showing replenishment of probiotics is needed in children with high caries risk.

The elevated SIgA concentration in probiotic group is seen at the T1 which reaches a plateau after T2 suggesting that intake of probiotic continuously elevates the rate of SIgA concentration during the first month. Thereafter, the SIgA concentration remain stable from week 4 until week 16 suggesting the continuous daily intake of probiotics may be efficacious for the maintenance of increased SIgA in the children

This was in accordance with **Caglar et al** who noted decreased level of S mutans level after 2 week use of L.reuteri enriched yoghurt and results were seen immediately during supplemention and for few days after discontinuation.[18] We utilized non sweetened milk as a vehicle to supplement probiotics which has additional health beneficial nutrients like casein,calcium and phosphorus.

Jorgensen et al stated no major alteration in SIgA concentration which is similar to our results however contradictory results showed an increase in SIgA concentration in saliva after ingestion of probiotic Bifidobacterium , Lactobacillus or Enterococcus strains.[6,14]. **Horz et al** found that the latency time of probiotic S.salivarius K12, by prescribing 4 tablets/day for 3 days to be 3 weeks in a 35 day followup study .[19]

Tormo Carnicer R et al reported oral intake of fermented milk L.casei DN-114 001 to induce non specific total SIgA in infant saliva because oral intake of b240 stimulates the gut mucosal immune system to enhance SIgA in saliva.[6,20]

Hackioja et al reported that consumption of yoghurt with L.rhamnosus probiotic on daily basis hosted this microorganism in the saliva for 3 weeks after stopping the intervention [21] while as **Yli-Knuuttila**[22] and his colleagues said that the same strain colonized the oral cavity temporarily and consistent consumption is needed for long term beneficial effect. The saliva flow rates show seasonal variation as **Takaoka et al** noted that whole saliva volume increases during spring ,reaches a plateau in summer and decreases from autumn to winter.[23]Our study was done from November to April(winter to spring) and we could get adequate unstimulated saliva.

The limitations of our study was the sample size was small and length of intervention with probiotics was short, probiotic sachet contained multiple bacterial strains along with L. Sporogenes which could have had a synergistic effect and the salivary samples should have been collected at an interval of one month each for a period of three months to correlate the immunostimulatory effect of probiotics on SIgA periodically.

Conclusion

Within the limitation of this study it can be concluded that probiotics have an immunostimulatory effect which is measurable in saliva. It is seen that probiotic (L.sporogenes) increases salivary IgA levels as long as the intervention is done however no long term effect is seen. Probiotic supplementation can temporarily enhance salivary IgA levels during the intervention period thereby decreasing further caries progression. However further research needs to be done to study the long term benefits of probiotic on the oral health.

Why this paper is important for pediatric dentistry?

- To study the correlation of salivary IgA and probiotic supplementation in school children with high caries index
- SIgA levels increased as long as probiotic enriched milk was provided however it didn't have a long lasting effect once the intervention was terminated.
- Probiotics have immunostimulatory and immunoprotective effect against caries thereby reducing its risk.

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