COMPARATIVE EVALUATION OF INHIBITORY EFFECT OF DENTRIFICE (PUREXA) AND MOUTHWASH(PERFORA) FORMULATED WITH PROBIOTICS ON SALIVARY STREPTOCOCCUS MUTANS LEVEL IN CARIES RISK POPULATION: AN IN-VIVO STUDY

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Article History: Received: 10.04.2023 Revised: 06.06.2023 Accepted: 01.07.2023 Published: 10.07.2023

Abstract

Introduction: Worldwide, dental caries is recognized as the most common microbial disease of complex etiology.The most important bacteria responsible for caries initiation is*Streptococcus mutans*. Studies in the field of caries prevention have shown that it is difficult to completely eliminate *Streptococcus mutans* from oral cavity by mechanical and chemical control only. Consequently, alternative ways to affect the oral ecology have emerged such as administration of probiotics which alters the bacterial ecology and antagonizes their growth.

Aim: The aim of the study was to evaluate the inhibitory effect of dentrifice (Purexa) and mouthwash (Perfora) formulated with probiotics on salivary *Streptococcus mutans* in caries-risk population.

Materials And Methods: 18-30 years old subjects were selected who underwent a second round of screening to determine the pH of saliva. 60 subjects with salivary pH <5.5 were included in the study which were then divided into 3 groups (n=20):

- Group A (n=20): Fluoride dentrifice without mouthwash (Control group)
- Group B (n=20): Probiotic dentrifice (Purexa) without mouthwash
- Group C (n=20): Fluoride dentrifice with probiotic mouthwash (Perfora)

All subjects were provided with necessary instructions regarding the use of the dentrifice and mouthwash. Two ml of unstimulated saliva was collected on the 1st day as baseline data and after 14 days of intervention and Colony forming Unit (CFU) of *S. mutants* on Mitis Salivarious Agar was calculated.Data was analyzed using Wilcoxon Signed Rank Test and Mann Whitney U Test.

Results: There was a significant reduction in *S. mutans*count in both the experimental groups as well as the control group after an intervention period of 14 days. However, probiotic dentrifice (Group B)and probiotic mouthwash (Group C) resulted in significantly more reduction in salivary *S. mutans* count when compared to subjects using only fluoridated dentrifice (Group A) with the highest reduction observed in Group C.

Conclusion: Administration of probiotics inhibited the growth of *S. mutans* and resulted in significant reduction of this caries-causing micro-organism. Early integration of probiotics in oral health regimens may go a long way in preventing the development and further progression of carious lesions and thereby serve as an invaluable caries-preventive measure.

Keywords: Caries prevention, Dental caries, Probiotics, *Streptococcus mutans*

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DOI: 10.31838/ecb/2023.12.s2.382

1. Introduction

The human oral cavity is known to harbor multiple micro-organisms which either exist in symbiosis or compete with each other for their nutrition and proliferation. [1] Worldwide, dental caries is recognized asthe most common microbial disease of oral cavity with a complex etiology, having a strong relation with the oral microflora. [2]

A carious lesion is initiated through a complex interaction over time between acid-producing bacterial population and fermentable carbohydrates.[3] The initiation of dental caries is believed to be caused by pathogenic strains of *Streptococcus mutans* and *Streptococcus sobrinus,* and its progression is brought about by *Lactobacillus spp.*[4]Over the years, different methods such as diet modification, fluoridated dental products and use of different oral hygiene aids have been advocated for the prevention of dental caries. However, it has become increasingly clear that measures directed at eradicating specific caries causing micro-organisms which are members of the endogenous flora have been proven difficult and maybe otherwise unwise. [5]

Consequently, alternative methods such as Bacteriotherapy or Replacement therapy which aims at altering the oral ecology by introduction of health promoting bacteria is gaining popularity.[6] The term probiotic, which means "for life," was first coined in the 1960s by Lilly and Stillwell.[7] According to the World Health Organization, probiotic bacteria are defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. [8] These probiotics competes with and antagonizes the growth of pathogenic bacteria and exerts health-promoting and therapeutic effects locally and systemically by influencing the immune system. [7]

Commonly, most of the species ascribed as having probiotic properties belong to the genera *Lactobacillus* and *Bifidobacterium*.[9]Existing clinical studies have demonstrated that lactobacilli-derived probiotics in dairy products may hamper the growth of salivary mutans streptococci.[10,11]However, dairy products (milk, yogurt, cheese, ice-cream) when used as vehicles for delivery of the probiotics have certain disadvantages such as irregular consumption and short shelf-life.[12]

Since maintenance of oral hygiene by regular brushing of teeth and use of mouthwashis an integral part of one's daily regimen, probiotic dentrifice and mouthwash were selected as preferred vehicles in this study and thepresent investigation was thereforeundertaken to evaluate the inhibitory effect of dentrifice (Purexa) and mouthwash (Perfora) formulated with probiotics on salivary *Streptococcus mutans* level in caries risk population.

2. Materials And Methods

Study Subjects

The study protocol was approved by the Institutional Ethics Committee of Bharati Vidyapeeth (Deemed to be University) Dental College and Hospital, Pune (EC/NEW/INST/2021/MH/0029).

Informed consent from the participantswere obtained before the commencement of the study.

The subjects were recruited from the patients who reported at the Outpatient Department (OPD) at the Department of Conservative Dentistry and Endodontics, Bharati Vidyapeeth (Deemed to be University) Dental College and Hospital, Pune. 18-30 years old subjects, who were healthy, non-smokers, did not have any other deleterious oral habits and had not undergone any caries preventive treatment in the last six months were included in the study. Subjects with a history of systemic antibiotic treatment, or habitual use of xylitol products or mouthwash within a 4-

week period prior to baseline investigation were excluded from the study.

Study Design

The selected subjects underwent a second round of screening using the Indicator Papers-Specific Range (pH 3.5 to 6.0), Qualigens Q38171 to determine the pH of saliva. (Table/fig-1) Subjects with a salivary pH of <5.5 were included in the study as they were more prone to develop dental caries.

60 subjects who met all the inclusion and exclusion criteria as well had a pH< 5.5 were selected and randomized into three equal groups and instructions for maintenance of oral hygiene was given.

- Group A (n=20) : Fluoride dentrifice without mouthwash (Control group)
- Group B (n=20) : Probiotic dentrifice without mouthwash
- Group C (n=20) : Fluoride dentrifice with probiotic mouthwash

Intervention

For the next 14 days, all subjects were provided with soft bristles toothbrush and were instructed to brush their teeth thoroughly twice a day for 2 minutes using their allocated dentrifice following a modified Bass brushing technique and were instructed to not eat or drink anything for the next half an hour. Subjects in Group C were asked to shake well and pour 10 ml (one cap) of undiluted Perfora Probiotic mouthwash, swish in the mouth for 30 seconds and spit out (according to the manufacturer's instruction). This was to be performed twice daily 30 minutes after toothbrushing and the subjects were asked not to eat or drink anything for the next half an hour. During the study duration, subjects were restrained from using any other dentrifice or oral product from another supplier and were instructed not to change their oral hygiene habits.

Probiotic dentrifice and Probiotic mouthwash

The Purexa Probiotic Toothpaste used contains 200 million CFU/gm helpful bacteria *Bacillus coagulans* (Probiotics) and is manufactured in Astonea Labs Private Limited, Haryana.(Table/fig-2) The Lemon Mint Probiotic rinse used in the study is an alcohol-free mouthwash containing *Bacillus coagulans* (Probiotics) (1.5 billion CFU per bottle) manufactured by Chipper Consumer Private Limited, Karnal, Haryana. (Table/fig-2)

Saliva sample collection and Laboratory Tests

Sampling of saliva was carried out between 9 and 10 a.m. before breakfast. Two ml of unstimulated saliva was expectorated directly into a graded test tube on the 1st day as baseline data and after 14 days of intervention. (Table/fig-3) The collected samples were transferred to the laboratory immediately in an ice box and*S. mutans* was isolated using the Pour Plate method.With a micropipette, 1 mlof the sample was taken and inoculated in freshly prepared Mitis Salivarious Agar and incubated at 37℃ / 48 hours for selective growth of *S. mutans*.

After 48 h of the incubation period,colonies were identified by gram staining and*S. mutans* appeared on the culture plate as small blue, round adherent colonies. (Table/fig-4) Colonies so identified were counted using a standardized digital colony counter(APHA Standard Method) and expressed as CFU/ml (Colony Forming Unit/ml).

Statistical Analysis

The data obtained were compiled on a MS Office Excel Sheet (v 2019, Microsoft Redmond Campus, Redmond, Washington, United States) and subjected to statistical analysis using Statistical Package for Social Sciences (SPSS v 26.0, IBM).Intra group comparison was done using Wilcoxon Signed rank test (up to 2

observations). Inter group pair-wise comparison was done using Mann Whitney U Test. For all the statistical tests, $p<0.05$ was considered to be statistically significant and $p<0.01$was considered to be statistically highly significant.

3. Results

The pre-intervention and post-intervention mean values of *S. mutans*countof all the groups are shown in Table/fig-5,6. The statistical analysis revealed that there was a significant reduction of *S. mutans*count in both the experimental groups as well as the control group after an intervention period of 14 days with the highest reduction seen in Group C followed by Group B and Group A. (Table/fig-5)
There was a statistically non-significant difference ($p>0.05$) seen for the mean values of *S. mutans*count between all the pairs of groups at baseline before the intervention. (Table/fig-7) However,after 14 days of intervention, the statistical analysis (Table/fig-7) revealed that subjectsusingprobiotic dentrifice (Group B)and probiotic mouthwash (Group C) showed considerably more reduction in salivary *S. mutans* count when compared to subjects using only fluoridated dentrifice (Group A) and this difference was found to be statistically highly significant. The results of the Mann-Whitney U test also revealed a statistically significant difference between the two experimental groups (Group B and C) with subjects in Group C showing the highest reduction in*S. mutans* level.(Table/fig-7) The intergroup comparison of mean values of *S. mutans* count at baseline and after 2 weeks of intervention is depicted in (Table/fig-8)

4. Discussion

With the emanation of more resistant strains, antibiotic resistance is becoming a more significant global issue. [13]Probiotic bacteria relies on the concept of microbial ecological change as an effective mechanism in preventing dental diseases. [14]The word ‘probiotic’ against ‘antibiotic’ has been introduced to inhibit, reduce or selectively remove pathogenic bacteria as well as prevent the emergence of resistant strains. [15]Probioticscompete with pathogens for attachment sites by altering the structure of salivary pellicle and thereby specifically prevents the adherence and further proliferation of caries causing micro-organisms. [12] Direct interactions with the dental plaque, inhibition of biofilm formation, modulation of plaque ecology, production of antimicrobial substances and indirect actions including regulation of mucosal permeability and modulation of systemic and local immune function are among the other proposed mechanisms of action of probiotics. [16]The present study was conducted to observe the inhibitory effect of dentrifice and mouthwash formulated with probiotics on salivary *Streptococcus mutans* level in caries risk population.
Studies have indicated that individuals with increased caries activity haveresting pH below 5.5. [17] Thus, in the present study, individuals with an increased risk of developing dental caries i.e., salivary $pH<5.5$ (which is detected by Indicator Papers-Specific Range Qualigens Q38171) were selected.
Previously Näseet al.[10] conducted a randomized, double-blind, placebo-controlled intervention study to examine the effect of milk containing L. rhamnosus GG on caries and the risk of caries in children when compared with normal milk and observed that the probiotic milk was able to reduce *S. mutans* counts and a significant reduction of caries risk was also seen.
According to the Centers for Disease Control and Prevention, using a toothbrush on a regular basis is crucial for preserving excellent dental health. [18] As maintenance of oral hygiene using

dentrifice and mouthwash are the most common oral hygiene methods, they were selected as preferred vehicles for daily delivery of probiotics. [19,20]

Saliva has traditionally been used as a diagnostic technique to assess a person's caries activity and risk [21,22] and therefore saliva samples were collected on the 1st day as baseline data and after 14 days of intervention to evaluate the *S. mutans*count.

According to a study by Dasanayake et al.[23], Mitis Salivarius Agar was found to be more sensitive in identifying Streptococcus strains.Hence, in the present study, freshly prepared Mitis Salivarious Agar was used for selective growth of *S. mutans.*

The results of this study showed that there were no significant difference in the baseline value of *S.mutans*among the groups prior to any intervention and therefore the effectiveness of these groups in reducing salivary *S. mutans* levels could be well-compared.

In the present study, a significant reduction of *S. mutans* levels was observed in both the experimental groups as well as the control group after an intervention period of 14 dayssuggesting that both fluoridated and probiotics formulations were effective in reducing the *S. mutans* count in saliva. However, probiotic dentrifice as well asprobiotic mouthwash showed significantly more reduction in Groups B and C when compared to fluoridated dentrifice (Group A).

There is considerable evidence that twice-daily use of fluoride toothpaste significantly reduces caries in young permanent teeth [24] and the result of this study is in accordancewith dozens of clinical trials, which support the significance of fluoride toothpaste usage for caries control. [25] The primary non-professional intervention to prevent caries is regular brushing with fluoridated toothpaste, however, fluoride concentrations in toothpaste affect the caries-preventive action,with larger concentrations associated with improved caries control.However, fluorosis (enamel deformities) in developing teeth is more likely to occur when toothpaste has a higher fluoride content. [26] Despite the fact that fluoridated toothpastes were once thought to be the gold standard for the prevention of dental caries, concerns have been expressed over dental fluorosis and the choice of fluoride toothpaste concentration should be balanced against the risk of fluorosis. [27,28] Theseconcernsassociated with the use of fluoridated toothpaste formulations have led to the search for innovative and effective alternatives.

One of the novel strategies for the prevention of dental caries entailsmodification of resident oral microbiotaand inhibition of pathogenic caries-causing strains by consumption of various probiotic formulations. [29] In our study, subjects in Group B and Group C used probiotic formulations containing *Bacillus coagulans*. Although traditional probiotics like Lactobacillus and Bifidobacterium species exhibit excellent probiotic properties, their survival rates are often low, ranging from 1 to 15%, with certain strains performing even worse. [30] However, *Bacillus coagulans*is a spore-forming probiotic bacteriumwhich is more tolerant of adverse environmental conditions than vegetative cells.Therefore, these spores can endure industrial manufacturing processes and have an extended shelf life over a wide range of temperature. [31,32]The significant decline in the *S. mutan*s count observed in Group B and Group C can be attributed to the fact that these *Bacillus coagulans*probiotics exert health benefits on the consumers.

The result of this study is in agreement with the study conducted by Jindal G. et al. who evaluated the effect of probiotics

on salivary *Streptococcus mutans* counts in Indian children and observed asignificant reduction in salivary mutans streptococci counts after 14 days of *Bacillus coagulans*probiotic ingestion suggesting that it can be a low-cost probiotic for preventing and treating dental caries in children. [33] Similar observations were made in the study conducted by Ratna Sudha Met al. [34]

In the present study, subjects in Group C showedincreased reduction of *S. mutans* level than subjects in Group B. This could be because of the synergistic effect offluoridated dentrifice inhibiting bacterial activity and enhancing remineralisation of enamel and the probiotic activity of *Bacillus coagulans*present in the mouthwash.

Jothika M et al. evaluated the efficiency of probiotic, chlorhexidine, and fluoride mouthwashes on plaque *Streptococcus mutans* level at four periodic intervals and observed all three mouthwash reduced *S. mutans* count. Mouthwash with probiotics was just as effective as those containing sodium fluoride and chlorhexidine and hence, probiotic mouthwash could be regarded as a useful oral hygiene regimen. [35]

Any measure aimed at preventing the earliest possible colonisation of pathogenic bacteria might be advantageous in the long run to prevent dental caries. [33] In the present study, effect of administering probiotics for only a brief period of time was evaluated. However, as this also resulted in significant reduction of pathogenic bacterial counts, it seems conceivable that continuous administration of probiotic preparation may have a preventative role against caries development.

5. Conclusion

Within the limitations of this study, it can be concluded that allgroups exhibited significant reduction of salivary *Streptococcus mutans*levelsafter a14 daysintervention period. However, administration of probiotics resulted in significantly more reduction of the pathogenic microorganism when compared to fluoridated formulations. Probiotics, by virtue of their natural therapy, appear to be a novel technique for caries prevention in light of an expanding global problem of antibiotic resistance that have shown to impede the effective treatment of microbial diseases. Therefore, early integration of probiotics in oral health regimens may go a long way in preventing the development and further progression of carious lesions and thereby serve as an invaluable caries preventive measure.

Limitations

The limitation of the study may belack of standardisation of the oral hygiene status of the patients which could have been preventedby performing oral prophylaxis for each patient.The patient's dietary habits were also not taken into account in this investigation.

Acknowledgement

The author wishes to express her gratitude to the faculty and colleagues of The Department of Conservative Dentistry and Endodontics, BVDU Dental College and Hospital, Pune for the constant help, support and valuable suggestions provided in making this manuscript.

Conflict Of Interest

The authors report no conflict of interest.

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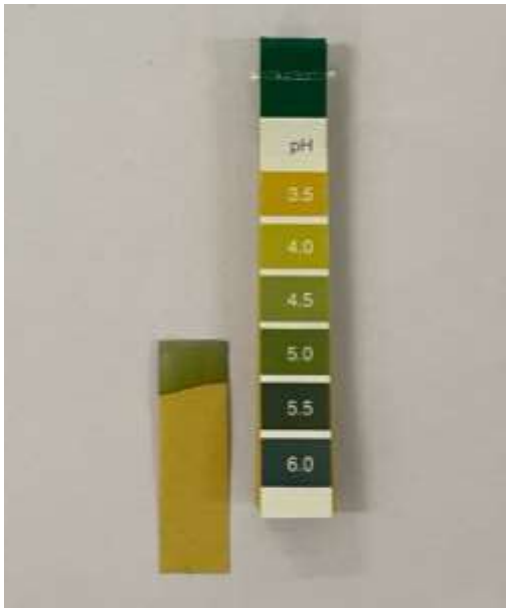
Tables/ Figures



Table/fig-1: (a)Measurement of pH of saliva (b) Indicator Paper – QualigensQ38171showing pH< 5.5

Table/fig-2 (a) Probioticdentrifice (Purexa) (b) Probiotic mouthwash (Perfora)

Table/fig-3: Sampling of saliva in a graded test tube

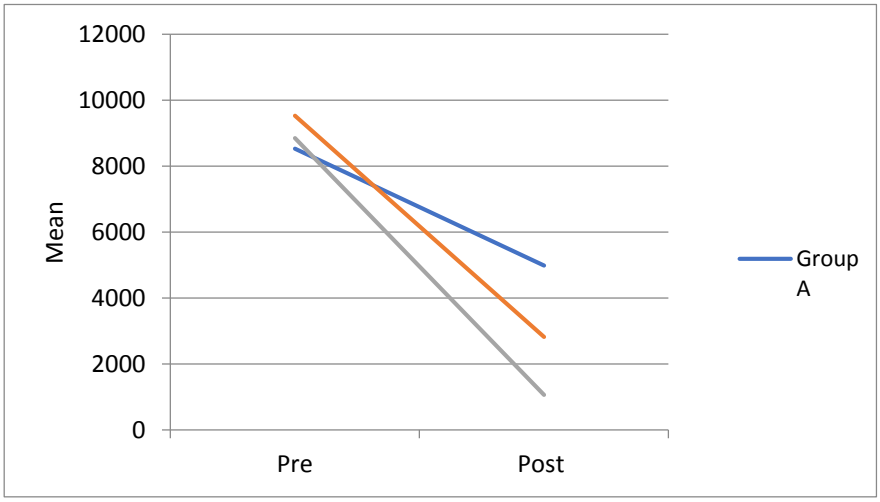
Table/fig-4: Growth of *Streptococcus mutans*in Mitis Salivarious Agar

Groups	Pre/post-intervention	Mean	Std. Deviation	Median	Mean diff	SD of diff	Z value	p value
Group A	Pre	8525.00	1826.667	8050	3540.000	1470.911	-3.921	0.000**
	Post	4985.00	2147.281	4650				
Group B	Pre	9530.00	3646.642	8600	6705.500	2883.533	-3.920	0.000**
	Post	2824.50	3200.058	870				
Group C	Pre	8843.50	3902.948	8550	7778.900	3717.986	-3.920	0.000**
	Post	1064.60	1566.869	450				

Table/fig-5: Intragroup comparison of mean values of *S. mutans*count at baselineand after 14 days of interventionusing Wilcoxon Signedrank test

* significant

** highly significant

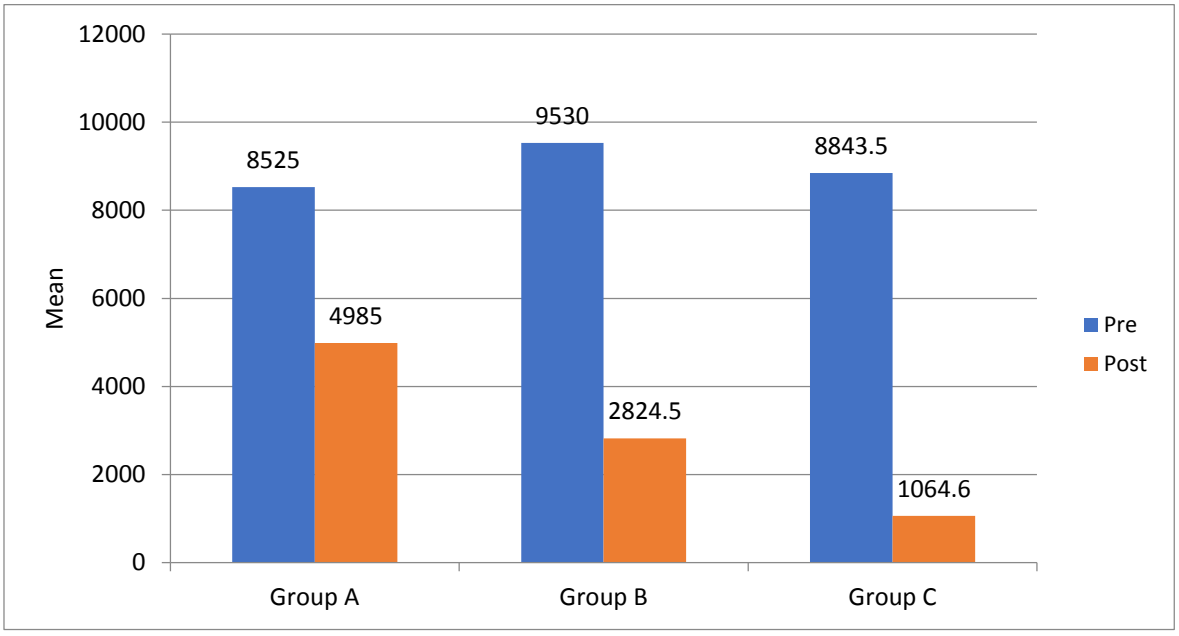


Table/fig-6: Line graph depicting the mean values of *S. mutans*count at baseline and after 14 days of intervention

Pre/post-intervention	**Group v/s**	**Group**	**Mann-Whitney U value**	**Z value**	**p value**
Pre	A	B	178.000	-0.596	0.551#
	A	C	190.000	-0.271	0.786#
	B	C	186.000	-0.379	0.705#
Post	A	B	102.500	-2.638	0.008**
	A	C	23.000	-4.789	0.000**
	B	C	119.500	-2.179	0.029*

Table/fig-7:Intergroup comparison of mean values of *S. mutans* at baseline (Pre) and after 2 weeks of intervention (Post) using Mann-Whitney U Test

* significant
** highly significant
\# non-significant



Table/fig-8:Graph depicting the intergroup comparison of mean values of *S. mutans* count at baseline and after 2 weeks of intervention

Tables/ Figures Legends

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- Table/fig-3: Sampling of saliva in a graded test tube
- Table/fig-4: Growth of *Streptococcus mutans* in Mitis Salivarious Agar
- Table/fig-5: Intragroup comparison of mean values of *S. mutans*count at baselineand after14 days of interventionusing Wilcoxon Signedrank test
- Table/fig-6: Line graph depicting the mean values of S. mutans count at baseline and after 14 days of intervention
- Table/fig-7: Intergroup comparison of mean values of S. mutans at baseline (Pre) and after 2 weeks of intervention (Post) using Mann-Whitney U Test
- Table/fig-8:Graph depicting the intergroup comparison of mean values of *S. mutans* count at baseline and after 2 weeks of intervention