Evaluation of Probiotic Effects of Lactobacilli on Mutans Streptococci: An In Vitro Study

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**ABSTRACT**

**Aim:** The aim of the present study is to evaluate the probiotic effect of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* on clinical isolates of Mutans Streptococci (MS) and antibiotic susceptibility of these strains to commonly used antibiotics in dentistry.

**Materials and methods:** Plaque samples from permanent first molars were collected and transferred aseptically onto Mitis–Salivarius agar. Plaque samples were then identified biochemically using the Hi-Strep identification kit. The inhibitory activity of the clinical strains of MS on Lactobacilli was investigated using agar-overlay inference technique. Positive inhibition was appreciated as a clear zone around the Lactobacilli. Disk diffusion assay was done as described by CLSI M100-S25 for antibiotic susceptibility. The zone of growth inhibition caused by Lactobacilli and antibiotics on MS clinical strains was measured directly using a vernier caliper. Statistical analysis was done using independent t-test.

**Results:** Mutans streptococci exhibited positive inhibition with both the probiotic strains and *L. acidophilus* showed more zones of inhibition than *L. rhamnosus*. Antibiotic susceptibility of clinical strains of MS showed sensitivity to penicillin and vancomycin, however, tetracycline and erythromycin showed very few resistant strains. The highest zone of inhibition was shown by cephalothin followed by penicillin, tetracycline, ciprofloxacin, erythromycin, and vancomycin.

**Conclusion:** *L. rhamnosus* and *L. acidophilus* have strong inhibitory effects on clinical strains of MS. *Lactobacillus acidophilus* showed a higher zone of inhibition. All the clinical strains were sensitive to penicillin and vancomycin. The highest zone of inhibition was shown by cephalothin.

**Clinical significance:** Dental caries remain silent epidemics and increasing antibiotic resistance is another major challenge that threatens the world. Newer methods such as whole-bacteria replacement therapy using probiotics for decreasing harmful oral pathogens and reducing the intake of antibiotics must be explored. More researches to promote use of probiotics should be initiated due to its possible preventive and health maintenance benefits providing an end to new cavities and antibiotic resistance.

**Keywords:** Dental caries, Lactobacilli, Mutans streptococci, Probiotics.

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**INTRODUCTION**

The human mouth is a complex ecosystem comprising more than 1000 bacterial species, contributing to the homeostasis in the oral cavity. Dental caries results when there is imbalance within this oral ecosystem.1,2 It is triggered by the interaction between caries-causing microorganisms, a diet rich in carbohydrates and host factors. The host factors are the tooth, immune response of the oral cavity, and the amount, consistency, as well as buffering capacity of saliva. The presence of all these elements leads to the initiation of the disease and the disease process can be intercepted by elimination of any one factor.3 Mutans streptococci was identified as the crucial pathogen in the commencement and further progression of dental caries.4,5 The acidogenicity of MS exacerbates the damage to dental hard tissues and its acidity contributes to its survival in low-pH environments.6

Imbalance between the number of indigenous bacteria to that of pathogenic strains can be highlighted as one of the major causes of dental caries. Hence, in order to nurture the equilibrium, competitiveness among bacteria appears to be a smarter approach to prevent the cariogenic flora from establishing its niche within the oral ecosystem and this may be achieved by probiotic therapy.2 Probiotics are live microorganisms, which when administered in adequate amounts, confer health benefits on the host.7 This concept is based on the idea of maintaining or restoring the natural microbiome in the oral cavity via interference or inhibition of pathogenic bacteria or both.8

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The management of dental caries since the beginning is largely concentrated on surgical methods, but today, as a profession, we are slowly seeing a transition from a purely surgical approach to minimally invasive dentistry with maximum consideration for prevention. With all the progression, science has furnished us with
the devices to diagnose and prevent the infection before it can cause damage. The strategic approach using probiotic therapy can bring about a total evolution in the prevention of dental caries.

The health benefits of probiotics recorded so far are prevention and treatment of infectious diseases, prevention of allergic disorders, preventing infections in hospitals, preventing necrotizing enterocolitis, severe pancreatitis, *Helicobacter pylori* infections, food allergies, preventing radiation and chemotherapy-induced diarrhea, allergic rhinitis, and improvement was also seen in patients with lactose intolerance. Probiotics are known to bring about control in blood pressure and cholesterol levels.⁹–¹¹ Among children, good results have been shown in the treatment of infectious diarrhea and allergic manifestations, in preventing antibiotic-associated diarrhea, otitis media, infections after vaccinations, respiratory infections, and urinary tract infections.¹²,¹³

Microorganisms identified as probiotic agents are chiefly the members of *Lactobacillus* or *Bifidobacterium* species and are gram-positive. They are generally regarded as safe.¹⁴ In recent years, the interest in probiotic therapies, especially Lactobacilli, to prevent and control dental caries, has grown significantly.³ The low-molecular-weight bacteriocin produced by Lactobacilli causes inhibition of MS. They can also hold themselves up to a pH of as low as 3.5 due to their acidic nature. This might be the reason for its increased interest among the researchers in prevention of dental caries.¹⁵ Several reports have demonstrated the therapeutic potential of Lactobacilli as probiotics in the prevention of dental caries in vitro, but studies on their effect on clinical strains of MS are minimal.¹⁶,¹⁷ In this context, the current study focuses on the probiotic inhibitory effect of *L. rhamnosus* and *L. acidophilus* on clinical isolates of MS. In the last few years, there has been an abundance use of over-the-counter medicines resulting in antibiotic resistance to commonly used antibiotics in dentistry. Hence, an antibiotic susceptibility test was also carried out for clinically isolated MS strains.

**MATERIALS AND METHODS**

The dental plaque samples to isolate MS strains were collected from the patients visiting the outpatient Department of Pedodontics and Preventive Dentistry of dental college from the time period of 2016–2017. The study was conducted upon obtaining approval from the Institutional Review Board and Ethical Committee (IEC/5/PEDO/MBDC/2015). The sample size derived was 40. Informed consent was also obtained from each participant.

**Sample-size Calculation**¹⁸

\[
n \geq z^2 \times p(1-p)/d^2.
\]

\(n\) – sample size, \(p\) – expected prevalence or proportion \((p = 63\%)\).  
\(d\) – allowable error (precision), \(d = 0.15\).  
\(z\) = statistic for a level of confidence, for the level of confidence of 95%, \(z = 1.96\).  
\[n = 1.96^2 \times 0.63 \times 0.37/0.15^2 = 40\]

**Selection Criteria**

Children between the age group of 6 and 12 years with DMFT/DMF scores of ≥3 were selected for the study. Children having antibiotics 1 month before the collection of samples, any probiotic supplements (mouth rinses/health drinks) for the last 6 months, and any anticaries substitutes (xylitol) for the past 2 weeks before the collection of samples were excluded from the study.

**Bacterial Strains for the Study**

*Lactobacillus acidophilus* MTCC 447T, *Lactobacillus rhamnosus* MTCC 1408, and *Streptococcus mutans* MTCC 495 were the bacterial strains used in the study that were procured from IMTECH Chandigarh. *Streptococcus mutans* MTCC 495 was used as the quality control for biochemical identification, antimicrobial susceptibility testing, and probiotic activity.

**Microbiological Sampling of the Patient**

Dental plaque samples from buccal, mesial, distal, lingual, and occlusal surfaces were collected using a sterile toothpick from the permanent first molar and transferred aseptically to sterile saline (Fig. 1).

**Microbiological Methods**

Plaque samples were vortexed for 1 minute and plated onto Mitis–Salivarius agar and incubated for 24 hours at 37°C in the presence of 5–10% CO₂. For the same, 40 plates were used. MS colonies were identified from the samples. MS strains produced “gum-drop” appearance with black centers. These colonies were subcultured onto blood agar for further identification using Hi-Strep biochemical identification kit (12 tests – sterile media for Voges Proskauer’s, Esculin hydrolysis, PYR, ONPG, arginine utilization tests, and 7 different carbohydrate utilization tests – glucose, lactose, arabinose, sucrose, sorbitol, mannitol, and raffinose) (Fig. 2).

**Fig. 1:** Microbiological sampling with paper points

**Fig. 2:** Clinical strains of mutans streptococci on Mitis–Salivarius agar
Agar-overlay Interference Technique

The standard strains of *L. rhamnosus* and *L. acidophilus* were cultured initially on MRS (deMan, Rogosa, and Sharpe) agar for 16–20 hours. Transfer of a distinct colony of *L. rhamnosus* and *L. acidophilus* was done to a 4.5-mL MRS broth and was further incubated for another 16–20 hours. The next day, pure colonies of *L. rhamnosus* and *L. acidophilus* were obtained. They were then transferred to 2-mL trypticase soy broth and were further incubated for another 16–20 hours. The media were incubated at 37°C with 5–10% CO₂. As described by Fleming, the inhibitory activity of the clinical strains of MS was investigated using agar-overlay interference technique. Briefly, the surface of MRS agar was spot-inoculated with 2 μL of an overnight culture of *L. acidophilus* and *L. rhamnosus*, adjusted to 0.5 McFarland. Agar plates were incubated at 37°C for 1 day in 5–10% CO₂ to allow colonies to develop. Then, they were overlaid with 7 mL of brain–heart infusion agar (0.75%), which was seeded with 0.1 mL of clinical strains of MS. After 48 hours of incubation at 37°C in 5–10% CO₂, positive inhibition was appreciated as a clear zone around the *L. rhamnosus* and *L. acidophilus*. Each experiment was done in triplicate.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility test of clinically obtained strains of MS was done using disc diffusion assay as described by CLSI M100-S25, for the following 6 discs on Mueller–Hinton agar supplemented with blood was taken. The antibiotics chosen were penicillin, tetracycline, ciprofloxacin, cephalothin, erythromycin, and vancomycin. Zone of Inhibition

The zone of growth inhibition caused by *L. rhamnosus* and *L. acidophilus* on MS clinical strains was viewed by inverting the agar plate and was measured directly using a vernier caliper. A similar procedure was carried out to measure the zone of growth inhibition caused by antibiotics on clinical strains of MS. Each experiment was done in triplicate and the average of inhibition was assessed.

Statistical Analysis

Descriptive statistics such as mean and standard deviation and inferential statistics such as independent t-test were employed in the present study. *p*-value ≤0.001 was considered statistically significant.

Results

Characterization of MS

The first 40 strains of MS were isolated from the plaque samples. MS strains produced a “gum-drop” appearance with black centers.

Growth Inhibition of MS

The result showed that all the 40 clinical strains of MS exhibited positive inhibition with the probiotic strains of *L. rhamnosus* and *L. acidophilus* (Fig. 5). The mean zone of inhibition of clinical MS strains by *L. rhamnosus* was 27.00 with SD 2.17 (Fig. 6). The mean zone of inhibition of clinical strains of MS by *L. acidophilus* was 27.58 with SD 1.95 (Fig. 6). The zone of inhibition shown by *L. acidophilus* was higher than *L. rhamnosus* and was statistically significant (*p*-value ≤ 0.001) (Fig. 6).

Antibiotic Susceptibility

The results showed that all the MS clinical strains were sensitive to penicillin and vancomycin (Fig. 7). Cephalosporins and cephalothin showed 92.5% sensitive and 7.5% intermediate strains (Fig. 7). Tetracycline showed 85% sensitive, 5% intermediate, and 10% resistant strains (Fig. 7). Erythromycin showed 67.5% sensitive,
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15% intermediate, and 17.5% resistant strains (Fig. 7). The mean and SD of zone of inhibition of clinical strains of MS by antibiotics are expressed in (Fig. 8). The highest zone of inhibition was shown by cephalothin followed by penicillin, tetracycline, ciprofloxacin, erythromycin, and vancomycin (Fig. 8).

Inference
All tested MS clinical strains showed positive inhibition with the probiotic strains of *L. rhamnosus* and *L. acidophilus*. The zone of inhibition shown by *L. acidophilus* was higher than *L. rhamnosus*. All the MS clinical strains were sensitive to penicillin and vancomycin. The highest zone of inhibition was shown by cephalothin followed by penicillin, tetracycline, ciprofloxacin, erythromycin, and vancomycin.

**Discussion**
The most common microorganism associated with dental caries is *Mutans streptococci*. They have essential cariogenic traits like glucan formation as well as aciduric and acidogenic properties. They also colonize on the surface of the tooth. The idea of using bacteria from the normal flora to combat harmful bacteria dates long back. In this era of minimal invasive dentistry, prevention of caries by colonizing caries-inhibiting bacteria like probiotic bacteria appears appropriate.

The beneficial effects of probiotic *Lactobacillus* species on general health are very well known. Hence, in the present study, Lactobacilli spp. were selected as they are generally regarded as safe. Some *Lactobacillus* spp. are known to cause tooth decay but this happens only when they overgrow in an acidic, oxygen-free environment created by MS. In a healthy mouth, they generate organic acids from carbohydrate fermentation, which could interfere, *in vivo*, with the growth of surrounding microorganisms by lowering the pH of the oral cavity. Additionally, some strains produce hydrogen peroxide or bacteriocins, which results in bacterial antagonism. They strengthen innate and acquired immunity as well as help in pro-inflammatory mediator’s inhibition.

*Lactobacillus rhamnosus* was selected for the current study as it has been used in the treatment of gastrointestinal infections. The ability of probiotic strain *L. rhamnosus* GG to adhere to salivary-coated hydroxyapatite has been shown in many studies *in vitro*. In order to establish colonization in the oral cavity, this adhesion, which results in prolonged retention of Lactobacilli species, is essential. The second strain selected in the present study was *L. acidophilus*. *Lactobacillus acidophilus* was preferred because of its acid resistance and bile salt’s tolerance. A number of compounds like organic acids, diacetyl, hydrogen peroxide, and bacteriocins, which have the ability to prevent dental caries, are produced by this bacterium. In the present study, both probiotics *L. rhamnosus* and *L. acidophilus* showed positive inhibition to all the clinical strains of MS tested. It was also noted that the zone of inhibition caused by *L. acidophilus* was higher than *L. rhamnosus* and was statistically significant.

The current study is in accordance with an *in vitro* study by Sookkhee et al., according to them, the selected probiotic strains of *L. rhamnosus* GG caused inhibition of *S. mutans*. This result may

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**Fig. 6:** The zone of inhibition of MS by *Lactobacilli*. The zone of inhibition of MS by *L. rhamnosus* and by *L. acidophilus*. The data is expressed in mean ± SD.* *p*-value ≤0.001 compared zone of inhibition of MS by *L. rhamnosus* and by *L. acidophilus*

**Fig. 7:** Antibiotic susceptibility of MS to antibiotics. Data expressed in percentage. Clinical strains of MS showed 100% sensitivity to penicillin and vancomycin. Cephalosporin’s and cephalothin showed 92.5% sensitive and 7.5% intermediate strains. Tetracycline showed 85% sensitive, 5% intermediate, and 10% resistant strains. Erythromycin showed 67.5% sensitive, 15% intermediate, and 17.5% resistant strains.
be due to interference of probiotics in biofilm formation and also antimicrobial activity of probiotics, which is pH-dependent. Raj et al. in an in vitro study showed that the isolates of L. rhamnosus have the ability to inhibit important oral pathogenic microorganisms, which also included S. mutans.

In another in vitro study by Jiang et al., the result showed L. acidophilus to be the best probiotic to inhibit biofilm activity of S. mutans and this was due to enzymatic inhibition of cariogenic bacteria and L. acidophilus showed more zones of inhibition when compared with L. rhamnosus. Similar results were seen in the present study too. The in vitro model of Tahmourespour and Kermanshahi showed that Lactobacillus species successfully integrated in all oral biofilms and reduced the adhesion of S. mutans. Similarly, a study by Demir and Demir showed that probiotic bacteria such as Lactobacillus spp. can affect the oral ecology.

Different studies by Widyarman et al., Nunpan et al., and Coqueiro et al. showed similar results stating that Lactobacilli can inhibit S. mutans. According to Nase et al., probiotics alter oral microflora and can be used as an adjuvant but cannot replace conventional treatment.

The findings of this study can be further justified by in vivo studies conducted on Lactobacillus species.

A randomized controlled trial was carried out in Finland by Ahola et al., they carried out a study on 1–6 years of age preschool children. In the experimental group, L. rhamnosus GG was added to the milk at their daycare centers 5 days a week for up to 7 months. A significant reduction in the counts of salivary mutants was seen. Similarly, Khanafari and Porgham showed the prophylactic effect of probiotics by daily intake of cheese containing L. rhamnosus LC705.

In a study by Salem et al., probiotic chocolate containing L. rhamnosus and L. acidophilus had an antimicrobial effect on S. mutans. When the mean zone of inhibition was compared between both of them, no significant difference was found. Similar study by Tehrani et al. showed that L. rhamnosus caused inhibition of S. mutans, which may be attributed to the presence of the antimicrobial agent but Bifidobacterium animalis caused no inhibition when S. mutans strains were tested.

Similarly, a study conducted by Chandak et al. on probiotic drops containing L. rhamnosus among children 3–6 years of age showed decreased salivary counts of S. mutans. Another study by Wu et al. showed similar results where L. acidophilus inhibited the growth of MS present in probiotic yogurt and L. rhamnosus present in pre- and probiotic capsules also inhibited S. mutans, but L. acidophilus showed more zones of inhibition than L. rhamnosus. The same results were seen in the present study also. Similarly, a study by Poorni et al. showed that commercially available yogurt inhibits S. mutans.

A systematic review by Shakib et al. included five articles that concluded that the application of oral probiotics will help reinstate a balanced microbiota, thereby improving oral health. Laleman et al. in their systematic review concluded that probiotic strains are able to control bacterial agents causing dental caries.

In the meta-analysis done by Sweeney et al., the comparison between the probiotic and control group after treatment showed significantly lower MS (<10^5 CFU/mL) counts among patients in the probiotic group and higher (>10^6) CFU/mL counts among control groups. The conclusion of the study was that the probiotics have the ability to decrease the MS counts, which in turn could have a positive effect in the prevention of caries.

With the emergence of resistant strains to antibiotics, it has become essential to perform routine susceptibility testing for MS strains. In the current study, we did antibiotic susceptibility test of clinical isolates of MS to commonly used antibiotics in dentistry. Large surveillance studies have demonstrated that MS has remained susceptible in vitro to penicillin, cephalothin, erythromycin, and vancomycin and few strains have shown resistance to tetracycline and ciprofloxacin. In the present study, antibiotic resistance was not significant and is in accordance with the study by Baker and Thornsberry and El Sherbiny. The clinical isolates of MS showed only 10% resistance to tetracycline and 17.5% resistance to erythromycin and were sensitive to all other drugs, the reason may be these drugs were priorly exposed to a particular drug or there might be a degree of immunosuppression, acquired resistant genes, or due to the intrinsic resistance of isolated MS strains. However, only one-time testing was done.
in our study; ideally, the study should be repeated with isolates at different time intervals from the same patients, so that any changes in the pattern of carriage and sensitivity to different antibiotics can be observed. In the current study, the highest zone of inhibition was shown by cephalothin followed by penicillin, tetracycline, ciprofloxacin, erythromycin, and vancomycin.55 The study by Ferretti and Ward showed similar results.57 According to the study by Baker and Thornsberry,45 Karikalan et al.36 penicillin is the most effective drug against MS.

The largest challenge faced by the world today is an increase in community- as well as hospital-acquired antimicrobial-resistant bacteria. The capability to efficiently treat patients is threatened. The solution is continuous surveillance, more appropriate antimicrobial prescription, and prudent infection control. New treatment alternatives should be emphasized.46 The use of bacteriotherapy using probiotics came as a unique concept for the prevention of dental caries and they also improve oral flora. The use of probiotics for dental caries is also noninvasive. The result from our present study as well as all the available literature support that probiotics can be an effective means to combat dental caries.58 However, it is necessary to create more awareness among the dentists and the general population, so that we can utilize bacteriotherapy that is a novel concept and can help to reduce dental caries. For the pediatric dentists, it is essential to know about all the preventive measures against dental caries as they are the one who deals with the children and encounters dental caries very frequently.

**Limitations of the Study**

In the current study, the isolated strains of MS from children were studied for inhibition by probiotic strains *in vitro*. The fact that this study is an *in vitro* investigation could not fully replicate oral environment and hence understanding of the complex triangular relationship of probiotics, pathogens, and oral microbiota within the oral cavity will be needed. More elaborated tests working with complex microbiota and environmental conditions similar to the oral environment are required to get a more accurate view of the anticarious properties of probiotics. Before making probiotics commercially available for clinical use, double-blind, randomized, placebo-controlled trials with specifically selected and defined strains are recommended. It may also prevent their spread into the environment and dissemination of the genetic modification.

**Conclusion**

Within the limitations of the present study, we can conclude that probiotics, especially *L. rhamnosus* and *L. acidophilus*, are effective in the prevention of dental caries.

The zone of inhibition of *L. acidophilus* was higher than *L. rhamnosus*. All the clinical strains of MS were sensitive to penicillin and vancomycin. The highest zone of inhibition was shown by cephalothin followed by penicillin, tetracycline, ciprofloxacin, erythromycin, and vancomycin.

**References**

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