ORIGINAL RESEARCH

Evaluation of Antibacterial Efficacy of Two Commercially Available Probiotics as Intracanal Medicament against *Enterococcus faecalis*: An *In Vitro* Study

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Abstract

Aim: This study was performed to evaluate the antibacterial efficacy of two commercially available probiotics (BIFILAC and VSL 3) as intracanal medicament against *Enterococcus faecalis* in endodontic therapy.

Materials and methods: Microorganisms from commercially available probiotics (BIFILAC and VSL 3) were extracted via the manufacturer's recommendations and mixed by weight. About 30 microliters were then placed on sterile discs. The pathogenic test organism was *E. faecalis* set to a 1 McFarland standard challenge. A two-probiotic disc template on blood agar plates was inoculated with *E. faecalis* and incubated at 37°C for 48 hours and 1 week respectively. Phase-1 of the study was conducted by a disc diffusion assay test to evaluate zones of inhibition (ZOI) in millimeters (mm). Phase-2 was conducted by mixing 9 mL of 30% poloxamer 407 and MRS broth in a test tube, together with the two probiotic mixtures and *E. faecalis*, set at a 2 McFarland standard. Serial dilutions up to 10^8 were done and the mixture was placed inside root canals and incubated at 37°C for 36 hours and evaluated for colony-forming unit (CFU)/mL counts.

Results: The results of phase-1 showed that probiotics *Lactobacillus rhamnosus* and *Bifidobacterium* species are effective in fighting against *E. faecalis* with the acceptable zone of inhibition. The results of phase-2 showed that both the probiotics are effective against *E. faecalis* with a reduction in the number of CFU after probiotic usage.

Conclusion: Commercially available probiotics can be used effectively as an intracanal medicament to fight against *E. faecalis*, Poloxamer 407 is a promising vehicle for delivering probiotics inside the root canal system. Further *in vitro* and *in vivo* studies are needed to determine the full potential of "Bacteriotherapy" with an application of probiotics.

Clinical significance: If probiotics are proved to be an effective intracanal medicament against *E.faecalis* they can be used as an alternative to calcium hydroxide as intracanal medicament with no side effects to the host.

Keywords: Antimicrobial, Bacteriotherapy, *Bifidobacterium,* Endodontics, Innovative, *Lactobacillus rhamnosus,* Probiotics , Zone of inhibition**.**

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Introduction

The success of root canal treatment depends on the three main principles, complete debridement of root canal system, disinfection, and three-dimensional obturation of the root canal system with a hermetic seal.[1] Even though the root canal procedures are done with the utmost care, certain factors like persistent intra-radicular and extra-radicular infections, foreign body reactions, and retained cysts lead to failure of root canal treatment.[2,3] According to various studies, the main factor that attributes to the failure of root canal treatment is the survival of microorganisms in the root-filled tooth.[1–3] The most commonly associated microorganism in the root canal failed teeth is *Enterococcus faecalis* is a gram-positive, commensal bacterium most commonly extracted from root canals of teeth with persistent endodontic infections. *Enterococcus faecalis* can survive as a single organism without the support of other bacteria inside the root canals and it can produce a biofilm. This necessitates the introduction of materials that will be able to reduce the number of disease-causing microorganisms in the apical portion of the root canal-filled teeth. One such invention is probiotics. The term Probiotics is derived from a Greek word meaning "for life" coined by Lilley and Stillwell in the year 1965.[4] Probiotics are the microorganisms that can inhibit the formation of pathogenic biofilm formation by competing with pathogenic micro-organisms for nutrition and increase their population by preventing the growth of pathogenic micro-organism. They are usually bacteria (beneficial microorganism) which are similar to the microorganisms found in the human body. Probiotics occur naturally in yogurts and fermented foods. They have various therapeutic and medical applications.[5] Traditionally probiotics are

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used in the treatment of gastrointestinal disorders, the probiotics are considered to have therapeutic effects by stabilizing gastric acid and bile salt secretion, adhering to the intestinal mucosa, and by colonizing the intestinal tract.[6] The studies evaluating the use of probiotics in the field of dentistry for the prevention of dental caries and periodontal diseases have proved that probiotics have the potential to reduce the growth of disease-causing microorganisms.[7] The use of probiotics in the field of dentistry is still evolving. The most commonly used probiotics in the field of dentistry are of the genus *Lactobacillus* and *Bifidobacterium*. These two micro-organisms are most abundantly seen in the oral cavity and carious lesions. They can be used as topical applicants. The oral administration of probiotics has been used to reduce plaque and thereby prevent dental caries and periodontal diseases. However, the usage of probiotics as an intracanal medicament in root canal therapy has not been studied extensively. So the purpose of this research is to evaluate the antibacterial efficacy of probiotics *Lactobacillus rhamnosus* and *Bifidobacterium* species over calcium hydroxide as an intracanal medicament against *E. faecalis*.[8,9]

Materials and Methods

Pathogenic Strain Selection

After ethical committee approval (2019-MDS-BrIV-HEM-10/APDCH) the study was carried out at Melmaruvathur Adhiparasakthi Institute of medical sciences (MAPIMS) from January 2019 to December 2020. The standardized microbiological protocol (ATCC guidelines) was followed to extract the probiotics and were done under strict aseptic conditions.[10,11] One gram of commercially available dried probiotic powder of Bifilac GG mouth melt vanilla granules, Tablets India Ltd containing *Lactobacillus rhamnosus*, and one gram of dried probiotic power from VSL 3 capsule Sun Pharmaceutical Industries Ltd containing *Lactobacillus* and *Bifidobacterium* species were measured on a lab scale separately and the measured powder of probiotics was added to 10 mL of MRS broth present in the conical flask. The tubes were then vortexed at room temperature on a vortex mixer (Fisher Scientific™ Digital mixer) for two minutes until the mixture was homogenous. The samples were then incubated at 37°C for 48 hours and kept in a lab refrigerator at 4°C to maintain the viability of the micro-organisms and also to avoid mutation in the test species. The prepared probiotic samples were used within two weeks. The study was conducted in two phases. Phase 1 was the discovery phase where the effect of probiotics was identified by evaluating the zone of inhibition (ZOI) of *E. faecalis* and the phase 2 was Application phase where the probiotics samples were mixed with vehicle poloxamer 407, and placed inside the root canal of extracted teeth, and colony forming unit (CFU) calculation was done.

Phase-1 (Discovery Phase)

Testing for Probiotic Efficacy against E. faecalis

A disc diffusion test was used to evaluate the efficacy of probiotics against *E. Faecalis*. The two test probiotic samples were extracted from commercially available tablets Bifilac GG Mouth Melt Vanilla Granules, Tablets India Ltd and VSL 3 capsule Sun Pharmaceutical Industries Ltd according to microbiologic principle and incubated for 48 hours. The probiotic samples were then placed in 9 mL of MRS broth (De Man, Rogosa, and Sharpe broth) and vortexed to ensure a homogenous mixture, then set to a 2 McFarland standard via laser spectrophotometry. The test micro-organisms *E. Faecalis* strain was received from ATCC and was kept in the refrigerator at 4°C. The storage of *E. faecalis* will lead to a reduction in the virulence of *E. faecalis*. To overcome the problem subculturing of the primary *E. faecalis* strain was done. The probiotic test sample was inoculated over blood agar plates where *E. faecalis* is uniformly dispersed, at the concentration of 50 µL (MIC) following the results of a pilot study evaluating the minimal inhibitory concentration of probiotics needed to fight against *E.faecalis*. The test samples were divided into three groups GROUP A – Probiotics Lactobacillus rhamnosus group, GROUP B – Probiotics *Lactobacillus* + *Bifidobacterium* species group, GROUP C – Calcium hydroxide group.

The agar plates were incubated at 37°C and evaluated at 48 hours and 168 hours respectively. The tests were conducted four times per group of probiotics to attain proper statistical analysis. Results of ZOI were measured with a digital micrometer in mm increments at 48 hours and 168 hours (1 week). The test results were statistically analyzed using the Kruskal–Wallis test to evaluate the efficacy of probiotic species against *E. faecalis*.

Phase-2 – (Application Phase)

Selection of Teeth

Ninety freshly extracted straight, single-rooted, single-canaled human permanent maxillary central incisors extracted for periodontal reasons with a curvature up to 10° with complete root formation and minimal root length of 18 mm were considered in the study. Conventional access cavities were prepared, and working length was established with the #15K file, instrumented using K-files up to # 25, followed by rotary files up to size F3 (ProTaper Universal, Dentsply, Switzerland). The canals were dried with absorbent paper points (Dentsply Sirona) and sterilized by autoclaving before the evaluation of the effect of probiotics against Enterococcus faecalis.

Testing of Probiotic/Pathogenic Organism Poloxamer Mixture Inside the Root Canal of Extracted Teeth

The biofilm staging was done by the evaluation of CFU after mixing pathogenic test micro-organisms, test samples, and intracanal delivery vehicle. The delivery vehicle used in this study was a poloxamer 407. The poloxamer 407 was dissolved in cold MRS broth at a concentration of 30% by using a magnetic stirrer for 15 minutes to obtain a homogenous mixture. To 9 mL of poloxamer-test micro-organism mixture the test samples were added. After 48 hours of incubation, serial dilution was made by adding 1 mL of poloxamer – test organism – test sample mixtures to 9.0 mL of sterile saline, followed by serially diluting the mixture by adding 1 mL of the previous mixture to 9 mL of sterile saline till 10^8 dilutions. The serially diluted poloxamer–test organisms–test samples were placed inside the root canals of the extracted teeth.

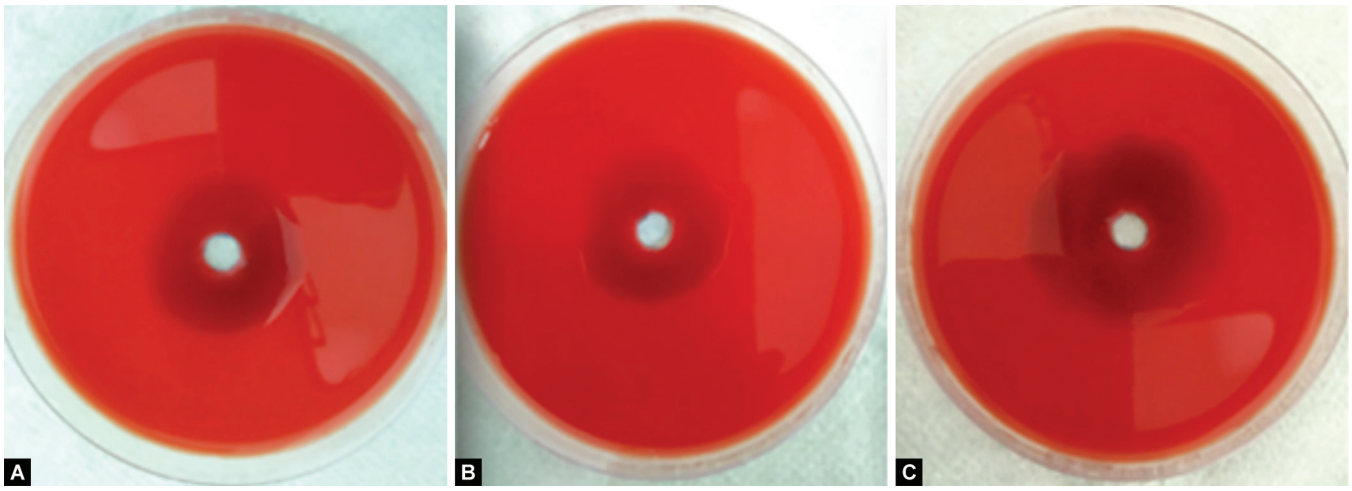
In group A *Lactobacillus rhamnosus* was placed as an intracanal medicament, In group B *Lactobacillus* and *Bifidobacterium* species were placed as an intracanal medicament, In group C calcium hydroxide was placed as an intracanal medicament, group D was the control group where poloxamer with no intracanal medicament was placed inside the root canal.

The teeth samples were incubated at 37°C for 72 hours in the incubator. Post incubation A sterile paper point was inserted into the root canal space to its full working length and was placed inside the canal for 60 seconds to extract the placed intracanal medicament mixture, CFU was evaluated for all test groups and

Table 1: Comparison of zone of inhibition based on time interval used using Kruskal–Wallis Test

	Lactobacillus rhamnosus		*Lactobacillus + Bifidobacterium species*		*Calcium hydroxide**		
Group	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*p-value*
48 hours	11.475	0.40311	10.0750	0.57373	18.1750	0.53774	0.007
1 week	12.1000	0.31623	10.5250	0.53774	18.4500	0.66081	0.007

*Calcium hydroxide 48 hours and 1 week- significant



Figs 1A to C: Zone of inhibition on blood agar plates after 48 hours: (A) *Lactobacillus* species; (B) *Lactobacillus + Bifidobacterium* species; (C) Calcium hydroxide

compared to controls based on the prepared dilutions to reflect the actual number of probiotics and pathogenic organisms in each group. The results were statistically analyzed using Wilcoxon signed-rank test to test the effect of the probiotics against *E. faecalis* species as intracanal medicament using poloxamer as an intracanal delivery vehicle.

Results

The data were analyzed for normality using the Kolmogorov-Smirnov test. The data were found to be non-normal in distribution. The intergroup comparison of the zone of inhibition were done using Kruskal–Wallis test. The intra group comparison of CFU pre and post-placement of intracanal medicament was done using Wilcoxon signed-rank test.

Interpretation for Comparison Zone of Inhibition among the Probiotics

Kruskal–Wallis test (Table 1) revealed that there was a statistically significant difference in the zones of inhibition between *Lactobacillus rhamnosus*, *Lactobacillus + Bifidobacterium* species, and calcium hydroxide at 48 hours ($p = 0.07$) and one week ($p = 0.07$). Calcium hydroxide showed the highest zone of inhibition at 48 hours and 1 week (18.17 ± 0.5 and 18.45 ± 0.6) when compared with *Lactobacillus rhamnosus* (11.475 ± 0.4 and 12.1 ± 0.3) and *Lactobacillus/Bifidobacterium* combination (10.07 ± 0.57 and 10.5 ± 0.53) (Fig. 1).

Interpretation for Comparison of Colony-forming Units before and after Placement of Probiotics

Wilcoxon signed-rank test revealed that there was a statistically significant difference in the CFU formed in *Lactobacillus rhamnosus*, preplacement (mean 1.6 ± 0.06), and post-placement (mean 0.16 ± 0.019) ($p < 0.05$) with the reduction in the CFU after probiotics placement inside the root canal. Overall lowest CFUs were seen in calcium hydroxide groups (0.08 ± 0.01) followed by probiotics *Lactobacillus rhamnosus* group (0.16 ± 0.019) and probiotics *Lactobacillus/Bifidobacterium* species combination group (0.35 ± 0.019) (Table 2).

Table 2: Comparison of colony-forming units before and after placement of probiotics using Wilcoxon signed-rank test

	Pre-placement		*Post-placement*		
Group	*Mean*	*SD*	*Mean*	*SD*	*p-value*
Lactobacillus rhamnosus	1.689	0.06855	0.165	0.01958	0.000
Lactobacillus + Bifidobacterium species	1.668	0.05073	0.359	0.01912	0.000
Calcium hydroxide	1.684	0.04719	0.089	0.01595	0.000
Control	1.684	0.06398	1.684	0.06398	1.000*

*Non-significant

Discussion

Traditionally root canal treatment involves mechanical instrumentation, irrigation, and irrigation activation devices such as sonics and ultrasonics to improve the effectiveness of cleaning and shaping.[12] Even after the introduction of various advanced methods in endodontic therapy, decontamination of the root canal system is still a challenging procedure. This is mainly due to the complex anatomy of the root canal system and the inability of the irrigants to reach the lateral canals and apical ramifications. This necessitates the introduction of new decontamination methods to completely eradicate all pathogenic organisms and thereby increase the success rate of endodontic treatment.[13] One such innovation is the use of

probiotics as antimicrobial agents inside the root canal system to fight against resistant endodontic pathogens.

The use of probiotics in the field of dentistry is still evolving. The most commonly used probiotics in the field of dentistry are of the genus *Lactobacillus* and *Bifidobacterium*. These two microorganisms are most abundantly seen in the oral cavity and carious lesions.[14]

Enterococcus faecalis are the most commonly isolated microorganisms in failed endodontic cases.[10] *Enterococcus faecalis* can survive as a single organism without the support of other bacteria and it can produce a biofilm. Due to the development of bacterial resistance by microorganisms, the use of antibiotics is being replaced by probiotics. According to Nase et al.[15] the most resistant strain *E. faecalis* can be eliminated by using probiotics. The probiotics produce various antimicrobial components like organic acids, hydrogen peroxide, bacteriocins, and adhesion inhibitors and thereby inhibit the growth of potentially pathogenic microorganisms.[1] This is the first kind of study that evaluated the effect of commercially available probiotics (Bifilac GG Mouth Melt Vanilla Granules, Tablets India Ltd, and VSL 3 capsule Sun Pharmaceutical Industries Ltd) against *E. faecalis* over calcium hydroxide. The results of the current study are in concordance with the study done by Seifelnasr, who compared five groups of commercial probiotics against *E. faecalis* and *Candida albicans* in two phases *in vitro*. The study results showed that organisms such as *L. acidophilus*, *L. rhamnosus*, *L. casei*, and *B. Longum* are effective in preventing the growth of *E. faecalis* and *C. albicans in vitro* against both their planktonic and biofilm morphological stages.[16] The results of the current study comparing the effect of probiotics at 48 hours and 1 week showed a statistically significant difference in the zone of inhibition for *L. rhamnosus* at 48 hours and one week ($p = 0.05$) suggesting sustained action of *L. rhamnosus* probiotics against *E. faecalis* for one week as intracanal medicament.

The preliminary study done by Arthi Bohora et al. in 2017 evaluated the role of two probiotics *Lactobacillus* and *Bifidobacterium* in endodontics based on the number and concentration of organisms. The pathogenic test organisms were *C. albicans* and *E. faecalis*. Two phases of the *in vitro* study were conducted. Phase 1 was conducted by the agar cup method to evaluate the antimicrobial activity of probiotics against *C. albicans* and *E. faecalis* by measuring the zone of inhibition in mm. Phase 2 of the study evaluated the CFU after serial dilution using 30% poloxamer 407 as a vehicle. The results of this study concluded that probiotic organisms of the species *Lactobacillus* and *Bifidobacterium* are effective in preventing the growth of *E. faecalis* and *C. albicans in vitro*. In the current study, the results of zone of inhibition for *Lactobacillus rhamnosus*, *Lactobacillus* + *Bifidobacterium* species, calcium hydroxide at 48 hours and 1 week, calcium hydroxide showed the highest the zone of inhibition and probiotics *L. rhamnosus*, *Lactobacillus* + *Bifidobacterium* species having an acceptable zone of inhibition which is similar to the results of the preliminary study done by Arthi Bohora et al.[17]

In the current study, the interpretation for comparison of colony-forming units before and after placement of probiotics was done by Wilcoxon signed-rank test. It showed a significant difference in the CFU formed in *L. rhamnosus*, pre, and post-placement. ($p < 0.05$) and also a significant difference in the CFU formed in *Lactobacillus* + *Bifidobacterium* species, pre, and post-placement ($p < 0.05$). The results of the study suggested probiotics as a good alternative to calcium hydroxide as an intracanal medicament to fight against *E. faecalis*. The results are similar to the results of a previous study done by Cosme-Silva et al.[18]

The evaluation of the effect of poloxamer as the delivery vehicle is the secondary goal of this study. Poloxamers are non-ionic polyethylene oxide and polypropylene copolymers which are biocompatible and can be used in various pharmaceutical formulations like surfactants, emulsifying agents, solubilizing agents, dispersing agents, and in vivo absorbance enhancers.[19] The results of this study showed poloxamer as a promising delivery vehicle. These poloxamers can be used as a delivery vehicle for placing probiotics in the future.

The concept of the human microbiome should be taken into consideration in endodontic therapy. The intentional establishment of microbial equilibrium in the root canal system by using probiotics may increase the overall success rate of apical periodontitis and also overcome the drawbacks associated with long-term use of calcium hydroxide. From previous studies, it has been established that probiotics can be used in the prevention of dental caries and periodontal diseases. The microorganisms in periodontal diseases are common in root canal infections. This shows the potential of probiotics in endodontics. The current study showed the effectiveness of probiotics in an *in-vitro* model. More studies reflecting the clinical scenario are required in the future to prove the effectiveness of probiotics in endodontic applications.

Conclusion

Commercially available probiotics can be used effectively as an intracanal medicament to fight against *E. faecalis*, Poloxamer 407 is a promising vehicle for delivering probiotics inside the root canal system. Further in vitro and in vivo studies are needed to determine the full potential of "Bacteriotherapy" with an application of probiotics. With fast-evolving technology and the integration of biophysics with molecular biology, designer probiotics pose a huge opportunity to treat diseases in a natural and non-invasive way.

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