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POTENTIAL ANTIBACTERIAL ACTIVITY OF PROBIOTICS AGAINST ENDODONTIC PATHOGENS: AN IN-VITRO PILOT STUDY

Dental Science

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ABSTRACT

Background: Primary goal of endodontics is to eradicate the infected pulpal tissues as well as the prevention of periapical infection. However failure to eliminate *Enterococcus faecalis* and *Candida albicans* is commonly associated with failed root canal treatment as well as acute and chronic apical periodontitis. In the light of human microbiome theory, this in-vitro innovative approach involves evaluation of antimicrobial efficacy of probiotics against these pathogenic organisms.

Methods: Antimicrobial activity of three commercial probiotics was evaluated against the test organisms i.e *E. faecalis* (ATCC 29212) and *C. albicans* (ATCC 10231). Agar disc diffusion method was employed to evaluate the antibacterial activity of the selected probiotics as well as their metabolic by-products against *E. faecalis* and *C. albicans* by measuring zones of inhibition (ZOI) in mm.

Results: With distinct zones of inhibition, two out of three tested probiotics as well as their metabolic by-products demonstrated the evidence of antimicrobial activity against both *E. faecalis* and *C. albicans.*

Conclusion: Within limitations of this in-vitro study, it is safe to say probiotics do possess ability to antagonize the growth of *E. faecalis* and *C. albicans*. This opens up a whole new avenue to use probiotics in treatment of endodontic infection.

KEYWORDS

Probiotics, endodontics, antimicrobial, drug delivery systems

INTRODUCTION

It was in the beginning of 20th century that a Ukranian bacteriologist and Nobel laureate Elie Metchnikoff first recognised the possible beneficial role of probiotics. Following which, in 1965, Lilley and Stillwell coined the term "probiotic". The word probiotic has its origin in Greek word meaning "for life".[1] With emergence of antibiotic resistance, use of probiotics is being constantly explored in the field of dentistry as an alternative to conventional antimicrobial agents.[2] In 2001, WHO proposed a definition for "probiotics" as living organisms, principally bacteria which when administered in adequate amounts confer a beneficial health effect, beyond the basic nutrition to the host. Commercially available probiotics contain strains of *Lactobacillus* and *Bifidobacteria* genus. Apart from these, certain strains of *Saccharomyces, Streptococcus, Enterococcus* and non-pathogenic strains of *E.coli, Clostridium butyricum* have also shown to possess probiotics properties.[3,4]

Field of endodontics aims to eliminate pathogenic bacteria by means of traditional chemo-mechanical methods. Reported success rates for enodontic therapy is up to 86–98%[5] which means failure ensues in substantial amount of patients (2-14%) when the endodontic treatment falls short of the standard clinical principles. *Enterococcus faecalis* and *Candida albicans* are almost always isolated and linked to the post-treatment endodontic failures.[6,7] Having proven beneficial effects of probiotics against periopathogens in our previous work[8], we aimed to extend the use of probiotics against these endodontic pathogens. The purpose of this study is to evaluate the potential antimicrobial activity of probiotic against enodontic pathogens i.e. *Enterococcus faecalis* and *Candida albicans.*

MATERIAL AND METHODS

Pathogenic strain selection

After a literature review in terms of most frequently isolated organism from failed enodontic treated cases is found to be *E. faecalis* ATCC 29212 from the Enterococcus genus. Thus it was chosen as one the test organisms. This organism does seem to play a vital role in terms of enodontic lesion.[9,10]

C. albicans ATCC 10231 is the most frequently associated fungus to the apical periodontitis cases associated with failed root filled teeth. Thus it was chosen as an another test organism for this study.[11]

Probiotic products:

Three different commercially available probiotic products were chosen. They were labelled as Probiotic-1[§], Probiotic-2[ǂ] and Probiotic-3[††]. Content of these probiotic samples are given in table 1.

Extraction of cell-free supernatant (CFS):

Probiotic samples 1, 2 and 3 were propagated in 100 ml flask containing MRS broth (pH 6.0) and incubated at 37°C for 72 hours under microaerophilic conditions. The CFS was obtained by centrifuging the culture at 10000 rpm for 20 minutes at 4°C. It is believed to contain metabolic by-products, antimicrobial substances like bacteriocin etc and a cell-free solution. Just to exclude any traces of bacterial cells, after centrifugation, the supernatant was filtered using Milipore membrane filter (0.45µ) and collected in a fresh sterile test tube.

Antimicrobial activity using agar disc diffusion method:

E. faecalis ATCC 29212 was inoculated on the blood agar plates while *C. albicans* ATCC 10231 was inoculated on sabouraud dextrose agar plates using lawn culture technique. Simultaneously, capsules of selected probiotics were then dissolved in three separate tests tubes containing 1 ml of sterile saline. To check the growth inhibition by diffusion method, three pre-sterilized non-impregnated paper discs[4] were then immersed in each probiotic solution and placed over already inoculated blood agar and sabouraud dextrose agar plates. A non-impregnated disc was placed at the centre of each plate as a negative control. Additionally, discs impregnated with Vancomycin was placed on *E. faecalis* ATCC 29212 inoculated blood agar plates and discs impregnated with Nystatin was placed on *C. albicans* ATCC 10231 inoculated sabouraud dextrose agar plates as positive controls. Tests were performed in triplicates.

In a similar fashion CFS of all three probiotics samples were tested for their potential anti-microbial activity. Both the test organisms were inoculated on their respective agar plates as described above. Three pre-sterilized non-impregnated paper discs[‡] were then immersed in each CFS solution and placed over already inoculated blood agar plates and sabouraud dextrose agar plates. Tests were performed in triplicates. All the plates were then incubated in an upright position at 37°C for 24 hours under aerobic conditions. Post incubation, zones of inhibition (ZOI) were measured in mm.

RESULTS

Agar Disc Diffusion Method using Probiotic samples (Fig.1) (Graph. 1):

A clear demarcated zone of inhibition around discs impregnated with Vancomycin on *E. faecalis* ATCC 29212 inoculated blood agar plates and around Nystatin impregnated discs placed on *C. albicans* ATCC 10231 inoculated sabouraud dextrose agar plates observed. This signifies presence and viability of these tested micro-organisms. For *E. faecalis* ATCC 29212 blood agar plate culture, 19±0.004 mm mean zone of inhibition around Vancomycin disc was observed. Whereas; 11.5±0.007 mm and 7±0.01 mm zones of inhibition were observed around Probiotic-1 and Probiotic-2 samples respectively. In case of *C. albicans* ATCC 10231 strain, 19.5±0.01 mm mean zone of inhibition was present around Nystatin impregnated disc. At the same time, Probiotic-1 and Probiotic-2 demonstrated 13±0.04 mm and 9±0.07 mm mean zones of inhibition respectively. Absence of well demarcated ZOI around Probiotic sample-3 against both *E. faecalis* ATCC 29212 and *C. albicans* ATCC 10231 strains suggests its lack of antimicrobial activity.

Agar Disc Diffusion Method using cell-free supernatant of Probiotic samples (Fig. 2) (Graph 2):

Similar trend was observed when cell-free supernatant (CFS) of all three Probiotic samples were tested for its potential antimicrobial activity. There were well demarcated zones of inhibition around discs impregnated with Vancomycin on *E. faecalis* ATCC 29212 inoculated blood agar plates and around Nystatin impregnated discs placed on *C. albicans* ATCC 10231 inoculated sabouraud dextrose agar plates. Vancomycin disc on blood agar culture plates for *E. faecalis* ATCC 29212 strain demonstrated 18.5±0.02 mm mean zone of inhibition. CFS of Probiotic-1 and Probiotic-2 samples showed 11.5±0.06 mm and 6.5±0.001 mm zones of inhibition respectively. For *C. albicans* ATCC 10231 cultures, 20±0.004 mm mean zone of inhibition was observed around Nystatin impregnated disc. Additionally, CFS of Probiotic-1 and Probiotic-2 samples demonstrated 12.5±0.005 mm and 8±0.04 mm mean zones of inhibition respectively. Again, with lack of well demarcated ZOI, CFS of Probiotic sample-3 showed no evidence of antimicrobial activity against both *E. faecalis* ATCC 29212 and *C. albicans* ATCC 10231.

DISCUSSION

In the era of antibiotics, overuse and abuse as well as rise in antibiotic resistance, use of probiotics can prove to be a beneficial alternative to most of the conventional approaches. Having already proven beneficial in the treatment several gastrointestinal diseases by restoring natural microflora, use of probiotics in the treatment of oral diseases is relatively a new modality.[12,13] Its use in treatment of various oral health related problems including gingivitis, periodontitis, halitosis, and caries prevention is extensively explored in recent times.[14,15] In the light of Human Microbiome theory, treatment approaches have moved from elimination of non-specific bacteria by use of antibiotic/antimicrobial agents or mechanical measures to maintenance/restoration of balance between the health promoting beneficial microbes by means of probiotics. In recent times, with wide spread emergence of antibiotic resistance due to the overzealous use of antibiotics, use of probiotic therapy can provide a natural and safe alternative to conventional treatment modalities. Adversely affecting growth of pathogens, either by competitive inhibition or by producing antimicrobial substances; actively limiting pathogens' ability of adhesion, colonization and biofilm formation on tissue surfaces or by immune-modulating the host; are few of the mechanisms by which probiotics act.[4] As a whole, probiotics are broadly classified in two genera i.e. *Lactobacillus* and *Bifidobacterium*. Several strains of these microbes have shown to possess probiotic properties. Most commonly studies strains are *L. acidophilus*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. reuteri*, *L. johnsonii*, *B. bifidum*, *B.longum*, *B. infantis*.[16]

Use of probiotics in treatment of endodontic lesions is a new approach not yet extensively studied. Conventional endodontic approach still revolves around mechnico-chemical removal of infected pulpal tissues to resolve pulpal and periapical infection. It is based on the premise that the pathogenic bacteria are the sole primary etiological factor for initiation and propagation of endodontic infection. Infection of pulpal tissue involves aggregation of a mixed bacterial community dominated by anaerobic gram bacteria. Additionally, persistent pulpal and peri-radicular infections as well as failed endodontic therapy is commonly associated with colonization of facultative anaerobic and gram positive microbe, in particular *E. faecalis*. Along with *E. faecalis*, *C. albicans* is the second most frequently isolated fungus from root filled teeth with apical periodontitis. This fungus having biphasic nature readily co-aggregates in forming complex biofilms that might propagate the infection further[17]. Complete elimination of *E. faecalis* is the biggest challenge as it is resistant to the most of the medicaments and has the ability to form complex bacterial biofilms known to resist penetration of these medications. This is the reason these particular organisms were selected for this in-vitro study.

Alternative to conventional antimicrobial approaches, growth inhibition by probiotics can be a potential avenue in endodontic therapeutic approaches. In this in-vitro study, growth inhibition by probiotics microorganisms as well as their metabolic by-products towards selected strains of *E. faecalis* and *C. albicans* is investigated. This in-vitro study aims at investigating possible application of bacteriotherapy to eliminate pathogenic organisms with the use of probiotic microbes either by employing their possible competitive inhibition mechanism or by antimicrobial substances such as peroxides, bacteriocins that they produce.[4]

This in-vitro study was able to demonstrate an active inhibition of tested endodontic pathogens by probiotics. Probiotic sample 1 and 2 were most efficient in antagonising growth of both *E. faecalis* and *C. albicans.* Amongst both, Probiotic-1 was most effective in terms of wider zone of inhibition. The difference in their inhibitory action could be due to difference in composition of the probiotics samples. A cell-to-cell, biochemical or molecular level interaction between probiotics and pathogens might be an explanation for this competitive inhibition of pathogenic microbes. The exact possible mechanism was out of scope of this study and it needs to be investigated further at molecular level using more advanced techniques. Growth inhibition of pathogenic microbes by probiotics seen in this study is in agreement with the previous studies.[18,19] Additionally, the cell free supernatant of Probiotic sample 1 and 2 both showed well demarcated zones of inhibition for both tested endodontic pathogens. This was most interesting fact as it might open up whole new avenue in terms of utilizing the extracted by products of probiotics instead of using probiotics themselves. Probiotic bacteria are known to produce many substances such as bacteriocin, peroxides etc which are harmful for the growth of pathogenic bacteria. This is important as even though probiotics are beneficial bacteria they can turn into pathogenic ones at larger doses or under certain conditions such as immune-compromised hosts etc.[20] Literature review suggests that most of the research is targeted on effect of probiotics against gastrointestinal bacteria. With promising results of this in-vitro study, we feel more studies should be directed against specific oral pathogens.

This pilot in-vitro study demonstrated that probiotics can play a vital role as an adjunct in endodontic therapy to maintain or restore equilibrium between pathogenic and beneficial bacteria. With shift in paradigm from complete elimination of pathogens by use of antimicrobial to replacing/restoring microbial-ecological equilibrium, use of probitics can open up whole new avenue. Probiotic bacteria can be utilized to counteract the growth of endodontic pathogens within infected root canal and to provide more conducive environment for beneficial bacteria to grow and restore the pulpal heath. More studies should be aimed at exploring possible systems to the sustained delivery of these probiotics strains to infected endodontic canal. Possible ways could be to combine with carrier molecules combined with probiotics spores. Alternatively, we feel their extracted cell free supernatant solutions can be used as irrigants to eliminate pathogenic organisms.

CONCLUSION

Within the limitation of study, preliminary data of this in-vitro study showed that probiotics as well as their metabolic by-products do have a potential to antagonize the growth of endodontic pathogenic bacteria. It opens up a whole new avenue for probiotics as an antimicrobial agent to counteract growth of endodontic pathogens. The results of this in-vitro study needs be validated further using larger sample size. More in-vitro, in-vivo research and large randomized trials are needed to

explore the possibility of using probiotics as an alternative to conventional endodontic therapy.

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Nil.

CONFLICTS OF INTEREST
There are no conflicts of interest.

FOOTNOTES
‡ HiMedia Laboratories Pvt. Ltd., Mumbai, India
§ EcoBion, Merck KGaA, Darmstadt, Germany
¥ Bifilac, TOA Pharmaceutical Co. Ltd., Tokyo, Japan
†† Ecoflora, Chr. Hansen, DK-2970 Hørsholm, Denmark

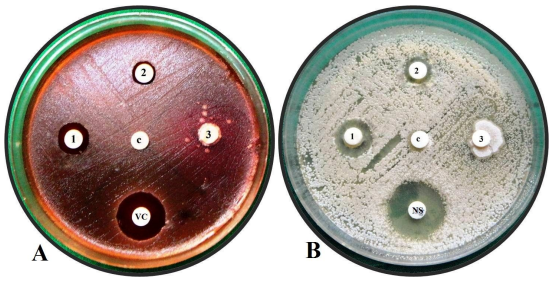


Figure 1. Antimicrobial activity of probiotics. A) *E. faecalis* ATCC 29212 inoculated blood agar plate B) *C. albicans* ATCC 10231 inoculated sabouraud dextrose agar plate. 1) Probiotic smaple-1, 2) Probiotic smaple-2 3) Probiotic sample-3 C) Control non-impregnated disc. VC) Vancomycin impregnated disc NS) Nystatin impregnated disc.

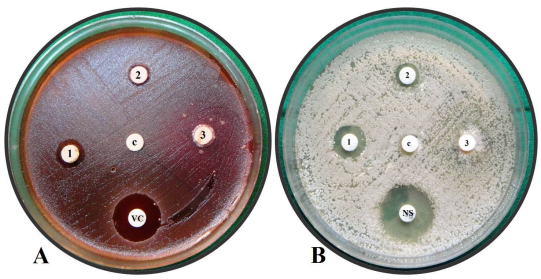
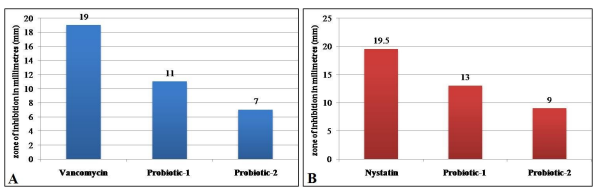
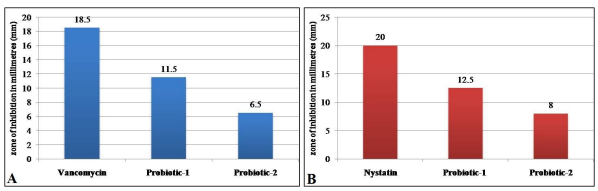


Figure 2. Antimicrobial activity of cell-free supernatant of probiotic samples. A) *E. faecalis* ATCC 29212 inoculated blood agar plate B) *C. albicans* ATCC 10231 inoculated sabouraud dextrose agar plate. 1) Probiotic smaple-1, 2) Probiotic smaple-2 3) Probiotic sample-3 C) Control non-impregnated disc. VC) Vancomycin impregnated disc NS) Nystatin impregnated disc.



Graph 1: Mean zone of inhibition in millimetres (mm). Antimicrobial activity of probiotics. against A) *E. faecalis* ATCC 29212 B) *C. albicans* ATCC 10231 strains



Graph 2: Mean zone of inhibition in millimetres (mm). Antimicrobial activity of cell free supernatant (CFS) of probiotics. against A) *E. faecalis* ATCC 29212 B) *C. albicans* ATCC 10231 strains

Table 1.

Probiotic-1	Probiotic-2	Probiotic-3
· Lactobacillus acidophilus (0.48 billion) · Lactobacillus rhamnosus (0.48 billion) · Bifidobacterium longum(0.48 billion) · Bifidobacterium bifidum (0.48 biilion) · Sacchromyces boulardii (0.10 billion) · Streptococcus thermophilus (0.48 billion) · + Fructo-Oligo saccharides (300 mg)	· Streptococcus faecalis T-110 JPC (30 million) · Clostridium butyricum TO-A (2 million) · Bacillus mesentricus TO-A JPC (1 million) · Lactobacillus sporogeneous (50 million)	· Lactobacillus rhamnosus Gr-1 (1 million) · Lactobacilllus reuteri RC-14 (1 million)

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