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Assessing the Antimicrobial Potential of Probiotics and Probiotic-Derived Supernatants against Enterococcus Faecalis: An in Vitro Analysis

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KEYWORDS

Enterococcus faecalis,Probiotics, Minimum, inhibitory concentration, Zone of inhibition

ABSTRACT:

Context:The primary goal of endodontics is to eradicate infected pulpal tissues and prevent periapical infections. However, the persistence of *Enterococcus faecalis* is commonly linked to failed root canal treatments and recurrent periapical infections..

Aim: To evaluate and compare the antibacterial efficacy of Probiotics and Cell free supernatant of Probiotics against *E. faecalis.*

Settings and Design: The antibacterial efficacy of the Probiotics and Cell free supernatant of Probiotics were assessed and compared *in vitro* against *E. faecalis.*

Methods and Material: The minimum inhibitory concentration (MIC) was determined using 96-well culture plates, and the optical density of each well was measured at 600 nm. The agar well diffusion method was performed to measure the zones of inhibition in millimeters.

Statistical analysis used: Data analysis was conducted using a one-way ANOVA test followed by post hoc analysis with Bonferroni correction.

Results: The MIC of probiotics against *E. faecalis* was optimized at 10 mg/mL. Probiotics and their metabolic by-products showed distinct zones of inhibition, demonstrating their antimicrobial activity against *E. faecalis*..

Conclusions: The results of this study suggest that probiotics and their cell-free supernatant could be incorporated into root canal medicaments, sealers, and irrigants, given their potent antimicrobial efficacy against *E. faecalis*.

1. Introduction

Dental caries is a chronic disease that can progress to pulpal and periapical pathosis which will be mutilating scenario that necessitates endodontic treatment. The success of endodontic treatment depends on the eradication of microbes from the root canal system and prevention of reinfection.[1] Control of infection is essential because the ample medullary bone spaces favor dissemination of infection and also because the developing permanent tooth germ is very close to the roots of the primary teeth. Thus, it is fundamental that the dentist be aware of the microbiota in these teeth so that adequate antimicrobial agents may be used to eliminate these pathogens.[2]

The micro-organisms associated with endodontic infections comprises of a complex mixture of bacterial species. *Enterococcus faecalis* is the one of the most predominant microorganism detected in root canals of teeth associated with persistent periradicular lesions. Its prevalence in primary endodontic infection is 40% and 24 to 77% in persistent endodontic infection. It is a non spore-forming, fermentative, facultative anaerobic,

Journal of Chemical Health Risks

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gram-positive cocci. They possess certain characteristics and virulence factors including lytic enzymes, cytolysin, aggregation substance, pheromones and lipoteichoic acid. *E. faecalis* has the ability to survive long periods of starvation, form biofilms, resist intracanal disinfectants, invade and live within the dentinal tubules for prolonged period of time and survive harsh conditions within root-filled teeth.[3] Historically, efforts to eliminate E. faecalis have been somewhat limited while using commonly used root canal irrigants.

With emergence of antibiotic resistance, use of Probiotics is being constantly explored in the field of dentistry as an alternative to conventional antimicrobial agents. 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.[4] The term "probiotic" was initially coined to oppose the term "antibiotic" by Lilley and Stillwell in 1965. 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.

Having already proven beneficial in the treatment of several gastrointestinal diseases by restoring natural microflora, use of probiotics in the treatment of oral diseases is relatively a new modality.[5] Its use in the treatment of various oral health-related problems, including gingivitis, periodontitis, halitosis, and caries prevention, has been widely explored in recent times. The application of probiotics in the treatment of endodontic lesions represents a novel approach that has yet to be extensively explored. Therefore, the aim of this in vitro study was to evaluate and compare the antibacterial efficacy of probiotics and their cell-free supernatant against *Enterococcus faecalis*, by determining the minimum inhibitory concentration (MIC) and measuring the zone of inhibition.

2. Methods

Enterococcus faecalis ATCC 29212, the most frequently isolated organism in failed endodontic cases, was chosen as the test organism for this study. The microtiter dilution assay was performed using Brain Heart Infusion (BHI) broth, according to the Clinical and Laboratory Standards Institute (CLSI) 2013 protocol. A probiotic solution was prepared by dissolving 20 mg of probiotic powder in 1 mL of sterile saline using an ultrasonic homogenizer. The test material was dissolved in 10% dimethyl sulfoxide (DMSO) and serially diluted in 96-well microtiter plates, with concentrations ranging from 500 µg/mL to 10 mg/mL. Each well was inoculated with 5 µL of a bacterial suspension containing 10^8 CFU/mL (0.5 McFarland standard) and measured at an optical density of 625 nm.

Positive controls consisted of BHI broth with the tested bacterial concentrations, while negative controls contained only inoculated broth. The plates were incubated for 24 hours at 37°C, and the optical density of each well was measured at 600 nm using a microplate reader. The minimum inhibitory concentration (MIC) was determined by visual turbidity before and after incubation, with each experiment performed in triplicate to confirm the results.

Commercially available probiotic powder (*Lactobacillus rhamnosus* - EnteroPlus, GLAXO) was used. A fluid form was prepared by dissolving 10 mg of probiotic powder in 1 mL of sterile saline, as this concentration was found to be the most effective MIC in this study. The solution was freshly prepared before use.

The probiotic sample was cultured in a 100 mL flask containing de Man-Rogosa-Sharpe (MRS) broth (pH 6.0) and incubated at 37°C for 72 hours under microaerophilic conditions. The cell-free supernatant (CFS) was obtained by centrifuging the culture at 10,000 rpm for 20 minutes at 4°C. To eliminate any remaining bacterial cells, the supernatant was filtered using a Millipore membrane filter (0.45 µm) and collected in a fresh sterile test tube.

The agar well diffusion assay was performed on Mueller-Hinton Agar (MHA) following the CLSI 2006 protocol. A sterile applicator moistened with the standardized cell suspension was used to inoculate the entire surface of the MHA plate, ensuring confluent growth. After the inoculum moisture was absorbed, two wells (6 mm diameter) were made in the plates at equal distances. Each well was filled with 20 µL of the test materials. The plates were incubated at 37°C for 24 hours. All procedures were performed in triplicate, and the zone of inhibition was measured in millimeters.

Journal of Chemical Health Risks

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The zone of inhibition around the well was recorded as a positive result. The values obtained were analyzed using a one-way ANOVA test, followed by post hoc analysis with Bonferroni correction. A p-value < 0.05 was considered statistically significant.

3. Results

The MIC of the probiotic solution against *E. faecalis* was determined using 96-well culture plates, with the optical density measured at 600 nm. The MIC was found to be effective at 10 mg/mL. [Figure 1] Subsequently, the antimicrobial efficacy of the probiotics and their cell-free supernatant was evaluated and compared using the agar well diffusion method. [Figure 2]

The mean percentage of inhibition of the test solution at various dilutions showed the highest inhibition at 10 mg/mL (89.91%) and the lowest at 500 µg/mL (39.85%), with this difference being statistically significant (p = 0.000). [Table 1]

Comparing the mean zone of inhibition, the cell-free supernatant demonstrated better antimicrobial efficacy (13.67 mm) compared to the probiotic solution (12.33 mm); however, this difference was not statistically significant (p = 0.133). [Table 2]

4. Discussion

The success of endodontic treatment is directly influenced by elimination of microorganisms in infected root canals. Failure to effectively eliminate them and their by-products might result in persistent irritation and impaired healing.[7] Numerous measures have been described to reduce the number of micro-organisms from the root canal systems, including the use of various mechanical instrumentation techniques, irrigation regimes and intracanal medicaments.[8]

E. faecalis is a facultative anaerobic, gram positive cocci, most predominantly found in persistent endodontic infections associated with root filled teeth. Its prevalence ranges from 24% to 77%. Various survival and virulence factors possessed by *E. faecalis,* include its ability to compete with other microorganisms, invade dentinal tubules, resist nutritional deprivation and survive very harsh environments extreme alkaline pH and salt concentrations. It has been shown to exhibit widespread genetic polymorphisms.[9] Thus it was selected as the test organism in the present study.

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.[10] Simply defined, probiotics are live bacteria that confer a health benefit to the host. The word probiotic has its origin in Greek word meaning “for life”. Probiotics organisms are classified by FDA as ‘generally regarded as safe’ (GRAS).

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.[11] Commercially available probiotics contain strains of *Lactobacillus* and *Bifidobacteria* genus. Most commonly studied strains are *L. acidophilus, L. rhamnosus, L. casei, L. paracasei, L. reuteri, L. johnsonii, B. bifidum, B.longum, B. infantis.*

Lactobacillus rhamnosus GG is a short gram-positive facultative anaerobic non-spore-forming rod that often appears in chains. In 1983, it was isolated from the intestinal tract of a healthy human by **Sherwood Gorbach and Barry Goldin**, the 'GG' derived from the first letters of their surnames.[12] *L. rhamnosus* has been one of the most widely studied probiotic strain, used in a variety of commercially available probiotic products. The beneficial effects of this strain have been studied extensively in clinical trials and human intervention studies and its use is considered safe,[13] because it has good characteristics of growth and adhesion in gut epithelium and this helps in competing with pathogenic micro-organisms on the gastrointestinal tract and intervening in immune system, intensifying the IgA production, stimulating the local release of interferons facilitating the antigenic transport to the lymphoid cells.[14] Hence it was selected as test material in the present study.

Even though probiotics are beneficial bacteria there are many concerns about their side effects like spreading of antibiotic resistance gene, virulence factor in particular strain, translocation to tissues or blood, risk of sepsis in premature infants, hindrance to normal colonization of other microflora.[15] They can turn into pathogenic ones at larger doses or under certain conditions such as

Journal of Chemical Health Risks

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immune compromised hosts etc.[16] Cell-free supernatants of probiotics aim to mimic the beneficial therapeutic effects of probiotics while avoiding the risk of administering live microorganisms to preterm infants with immature intestinal barriers or impaired immune defenses.[17] Cell-free supernatants (also known as Postbiotics or Metabiotics) refers to the metabolic by products like enzymes, peptides, teichoic acid, peptidoglycan derived muropeptides, exopolysaccharides, cell surface and secreted proteins, bacteriocins and organic acids generated by a live bacteria or released after bacterial lysis.[18] Thus Cell free supernatant of Probiotic solution was used as a test material in the present study.

Routine microbiological tests such as MIC and Agar diffusion tests were performed. MIC method uses serial dilutions of a solution to determine the lowest concentration of material that would still show antibacterial properties. The MIC is the lowest concentration of antimicrobial agents that visually inhibits 90% growth of microorganisms.[19] The MIC of Probiotic solution against *E. faecalis* was determined and was found to be effective at 10mg/ml. Agar well diffusion test has been used to evaluate and compare the effectiveness of the test material against *E. faecalis*, and the diameter of the microbial inhibition zone was measured. Cell free supernatant of Probiotic solution had better antimicrobial efficacy than Probiotic solution against *E. faecalis* in the present study and the results were similar to the studies done by **Joshi CP et al (2017)[6]** and **Bohora A and Kokate S (2017)[20]** and however the association was statistically non significant.

Cell free supernatants contain various metabolites and signalling molecules which display broad antibacterial spectrum and immunomodulatory actions.[21] The cell-free extracts from lactic acid bacteria may exhibit significantly higher antioxidant capacity than whole cell cultures.[22] They are supposed to be more stable than the living bacteria they are derived from.[17] They have advantage due to their clear chemical structure, safety dose parameters and longer shelf life which can influence physiological function of host.[23] These could be the most probable reason for the better antimicrobial activity of Cell free supernatant than Probiotic solution in the present study.

The protocol for the Probiotic medicament would be, instead of a microbial "elimination therapy" by the use of antimicrobial agents, substituted by a microbial "replacement therapy" allowing a more favorable/biocompatible environment using it as an intracanal medicament. In addition to eliminating and outcompeting the pathogens that originally entered from the carious process, probiotic organisms could not only eliminate disease causing bacteria but might also prevent their re-establishment after treatment has been completed, thus decreasing the incidence of endodontic failure.[24]

6. Conclusion

Within the limitations of this in vitro study, we conclude that using probiotics as an antimicrobial agent against *E. faecalis* is feasible. Incorporating probiotics into intracanal medicaments, root canal sealers, and irrigating solutions could effectively eradicate *E. faecalis* from the root canal, thereby enhancing the success rate of root canal treatments. Although the difference in antibacterial activity between the cell-free supernatant and the whole probiotic solution was not statistically significant, it is advisable to use the cell-free supernatant in clinical settings due to its advantages over the whole probiotic solution. Despite these promising results, further validation is necessary through additional in vitro and in vivo studies, as well as large randomized trials exploring various probiotic strains, to establish the potential of probiotics as an adjunct to conventional endodontic therapy.

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