Antimicrobial activity of three different Probiotic strains and 5.25% Sodium hypochlorite against E.faecalis and C.albicans at two different time period: An in-vitro study

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Abstract- AIM: The aim of this study was to evaluate and compare the antimicrobial efficacy of three different probiotic strains and 5.25% Sodium hypochlorite against E.faecalis and C.albicans at 48 hour and 1 week.

MATERIALS AND METHODS: Three commercial probiotic strains (L. acidophilus, L. Casei and L. Rhamnosus), 5.25% sodium hypochlorite and normal saline were selected and evaluated for zone of inhibition (ZOI) and colony forming unit (CFU). Pathogenic test organisms used were Candida albicans and Enterococcus. faecalis. Phase 1 of the study was conducted by a disc diffusion assay test to evaluate ZOI in millimetres (mm) of the selected groups against C. albicans and E. faecalis for 48 hours and 1 week. Phase 2 was conducted to evaluate CFU/ml counts after samples were incubated at 37°C for 72 hours.

RESULTS: Intergroup comparison of mean ZOI showed statistically significant difference among all tested groups for both the microorganisms and at both time intervals. The maximum ZOI was found in Group 4 (5% NaOCl) and minimum in Group 5 (Normal Saline). The mean CFU/ml counts amongst the groups was found to be highest in Group 5 (Normal saline) against both the microorganisms and lowest in group 2 (Lactobacillus Casei) against C.albicans and in group 3 (Lactobacillus. Rhamnosus) against E.faecalis.

CONCLUSION: This study suggests that Probiotic strains such as L. acidophilus, L. Rhamnosus, and L.Casei are effective for preventing the growth of E. faecalis and C. albicans.

Index Terms- Antimicrobial activity, L. acidophilus, L. Casei, L. Rhamnosus, Probiotic strains, 5.25% sodium hypochlorite, Zone of inhibition

I. INTRODUCTION

Factors that may contribute to a persistent periradicular infection after root canal treatment include intraradicular infection extra radicular infection, foreign body reaction, and cysts containing cholesterol crystals.[1] The main cause of failure is generally believed to be the survival of microorganisms in the apical portion of the root - filled tooth.[1-2]

Many studies have shown Enterococcus faecalis to be the most common bacteria isolated from teeth with failed root canal treatment.[3] The pathogenicity of E.faecalis has been attributed to its inherent antimicrobial resistance, increased virulence factors such as adherence to host cells[4], expression of proteins to ensure cell survival as a result of altered environmental nutrient supply[5], adherence to collagen in the presence of serum and ability to form calcified biofilm within the root canal.[6, 7]

Candida albicans is another microorganism that has been regularly identified in teeth with persistent apical periodontitis after treatment.[8] It can survive as a monoinfection and even invade dentinal tubules.[9]

The ultimate goal of endodontic treatment is to remove bacteria, there by - products and pulpal residues from infected root canals and the entire seal of disinfected root canals.[9] Among the available irrigants, sodium hypochlorite (NaOCl) has been accepted as the main irrigants during the treatment of the root canal for decades[10] and used at different concentrations (0.5% to 5.25% or higher). Its main property is the ability of dissolving organic tissue[11]. Sodium hypochlorite is effective in killing bacteria, spores, yeast and virus in vitro.[12]

Probiotics have recently been introduced in dentistry for treatment or disease prevention. “Probiotics" as defined by the World Health Organization are live microorganisms that provide the host with a health benefit when administered in adequate quantities. Bacteria or yeast may be probiotics, but most probiotics are bacteria. Bacteria with lactic acid are more popular among bacteria.[13-14]

The most commonly used strains of probiotic bacteria belong to the genera Lactobacillus. Species of Lactobacillus help to produce enzymes that digest and metabolize proteins and carbohydrates. Over 100 species of L. Rhamnosus, L. Acidophilus and L. Casei was identified.[13-14]

The potential use of probiotics for oral health has recently attracted several researchers’ attention. Although only a few clinical studies have been carried out to date, the results suggest that probiotics may be useful in the prevention and treatment of oral infections, including dental caries, periodontal disease and halitosis.[15]

To the best of our knowledge probiotics pertaining to L.Rhamnosus and L. Casei has not been used against E.faecalis and C.albicans.

Therefore, this study was carried out to assess and compare the effectiveness of three different probiotic strains and sodium hypochlorite against E.faecalis and C.albicans.

The null hypothesis was that different probiotic strains used in this study are not effective in inhibiting the growth of E.faecalis and C.albicans.

II. Materials and Method

For the present microbiological study, three commercial probiotic strains and 5.25% Sodium hypochlorite were purchased for utilization in this study.

The probiotic were delivered in wrapped ice packaging in order to preserve viability of the organisms. Upon arrival, the probiotics were stored in a refrigerator at 30º F.

For evaluating zone of inhibition and colony forming unit counts following groups were selected:
Group 1: Lactobacillus Acidophilus
Group 2: Lactobacillus Casei
Group 3: Lactobacillus. Rhamnosus
Group 4: 5.25% Sodium Hypochlorite
Group 5: Normal Saline

Pathogenic strain selection

Enterococcus faecalis ATCC 29212 was chosen for this study after extensive literature review, which revealed that this organism possesses multiple properties leading to its key role as an endodontic pathogen.

Candida albicans ATCC 10231 was chosen as another pathogenic test organism because of its biphasic nature, which allows it to be the universal co-aggregate in biofilms; it is the most frequently isolated fungus from root filled teeth withapical periodontitis.

Phase 1: Testing for Probiotic, 5.25% sodium hypochlorite and normal saline efficacy against E.faecalis and C.albicans:

Lactobacillus strain was isolated from each of the commercially available probiotic .These strains were further evaluated using different microbiological techniques. The three probiotic groups were extracted according to manufacturer's instructions and incubated for 48 hours. Probiotic samples were then placed in 9ml MRS broth (De Man, Rogosa and Sharpe Broth) and vortexes to insure a homogenous mixture and then set to a 2 McFarland standard.
The pathogenic organisms *E. faecalis* and C. *Albicans* were freshly stocked, placed in 9ml MRS Broth vortexes to insure homogenous mixture, then set to a 1McFarland standard.500 microliters of *E. faecalis and C. Albicans* was plated on 100 mm diameter blood agar plates and Spread with a sterile L-Loop. The sample was incubated for 24 hours to allow growth of a bacterial lawn.

Agar Cup Method/Disc diffusion method:

Twenty microliters of prepared solution of each test Groups 1,2,3,4 & 5 were placed on filter discs and left for 15 seconds to allow the discs to saturate with the probiotics strain, 5.25% sodium hypochlorite and normal saline on sterile blank paper. The discs were then transferred to the previously grown lawns of the pathogenic test organisms, E.faecalisand *C. albicans*, according to a 5 group template. The test was conducted three times per group against the organisms, *E. faecalis* and *C.albicans* to allow proper statistical analysis. Results for ZOI's were measured with a digital micrometre in mm increments at 48 hours and 168 hours (1 week).

Phase 2: Intra-canal delivery vehicle for probiotics:

The Poloxamer was dissolved in cold MRS broth at a concentration of 30% by a Magnetic stirrer for 10 to 15 minutes until a homogenous mixture was obtained. The Poloxamer was then sterilized and placed in the refrigerator at 4° C until testing was conducted. The MRS broth mixture was utilized for this study.

Testing for probiotic/pathogenic organism Poloxamer mixture:

Serial dilutions were made by adding 0.1 ml of Poloxamer mix to 9.9 ml sterile saline, followed by serially diluting the mixture by 0.1ml into 9.9 ml sterile saline three times, reaching dilutions of 10^{-2},10^{-4},10^{-6} respectively. Plating was conducted by adding 500 ml of dilutions onto blood agar plates, followed by incubation at 37° C for 72 hours. CFU (colony forming units) were evaluated for all test groups (groups 1 to 5) and compared to controls based upon the dilutions that were performed to reflect the actual number of probiotics and pathogenic organisms in each group.

Data was analyzed using SPSS version 21. Intergroup comparison was done using Kruskal Wallis test along with post hoc pairwise comparison by Mann Whitney U test. Intragroup comparison was done using Wilcoxon signed rank test. The level of statistical significance was set at 0.05.

III. Results:

Table 1 shows intergroup comparison of mean zone of inhition (ZOI) against C.albicans and E.faecalis at 48 hours and 1 week.

On comparing ZOI between groups at 48 hours and 1 week against C.albicans and E.faecalis, the maximum ZOI was found in Group 4 (5 .25% NaOCl) and minimum in Group 5 (Normal Saline). Intergroup comparison of mean ZOI showed statistically significant difference among all tested groups for both the microorganisms and at both time intervals.

Table 2 & 3 shows intergroup comparison of mean number of colony counts against C.albicans and E.faecalis respectively.
The mean number of colony counts amongst the groups against C.albicans was found to be highest in Group 5 (Normal saline) and lowest in group 2 (Lactobacillus Casei).

Similarly, the mean number of colony counts amongst the groups against E. faecalis was found to be highest in Group 5 (Normal saline) and lowest in group 3 (Lactobacillus. Rhamnosus).

Intergroup comparison of mean CFU/ml counts against C.albicans and E.faecalis showed that there was statistically significant difference amongst all the groups.

Intragroup comparison of mean zone of inhibition (ZOI) against E faecalis & C.albicans at 48 hours and at 1 week was statistically insignificant.

IV. Discussion:

It is known that microorganisms in infected root canals include a limited group of species compared to the rest of the normal flora found in the oral cavity. Enterococcus faecalis andCandida albicans are bacterial strains consistently found in endodontic disease after treatment.[1-3]

In order to maximize the disinfection of the root canal system in infected cases, in particular in the treatment of endodontically treated teeth, the use of intracanal drugs can help reduce the remaining microorganisms and provide an environment conducive to the repair of periapical tissue.[16]

An antimicrobial agent that does not cause toxic effects on periapical tissues must be the ideal irrigation solution.[17] Probiotics have recently been introduced in dentistry to control the growth of some oral microorganisms, including cariogenic species associated with dental caries.In the control of periodontal disease, the oral administration of probiotics was also investigated by reducing plaque levels and inflammation of the gingival.[15-16] The most commonly used bacterial strains of Probiotic belong to the genera Lactobacillus and Bifid bacterium.[11-16]

In this study, three different probiotic commercial strains of L. acidophilus (Group 1), L. Rhamnosus (Group 2) and L. Casei (Group 3) were used.

In the present study normal saline was completely ineffective against E.faecalis and C.albicans at both time periods. This finding was similar to Bohora and others findings[17]. As an antimicrobial agent, they found saline to be completely ineffective.

The null hypothesis was rejected in the present study, since all three Probiotic strains (L.acidophillus, L.Rhamnosus and L. Casei) inhibited the growth of E faecalis and C Albicans at both time periods.

The reason could be due to release of organic acid by probiotics into the bacterial cell and disrupting the regular metabolism. They also produce hydrogen peroxide that causes cell wall rupture and bacteriocins that inhibit DNA and other protein structures.[18] Geier[19] reported that Probiotic kills pathogens by producing bacteriocins and acids / peroxides and altering the pH of the local environment. Probiotics also compete with carbohydrates required for metabolism and remove gram - negative bacteria from the organism by producing fatty acids with toxic effects.[18] Fatty acid can disrupt the external membranes of gram - negative pathogens that inhibit the growth of pathogen.[20-22]

Till date very few studies have been done were Probiotics were checked against endodontic pathogens.

Similar to our results, Seifelnasr[23] found three probiotic strains in their study, i.e. L.acidophillus, Rhamnosus and Casei to be effective against E faecalis and C Albicans. Contrary to our results, Hammad[24] has shown that lactobacillus strains have no inhibitory effect on E.faecalis. Montecinos and others[25] examined the relationship between Rhamnosus probioticstrain during the Biofilm formation. They found that probiotic strain interfered in vitro with the E faecalis biofilm formation, thereby intensifying the growth of E. faecalis biofilm. Bohora and others[26] and Mcgroarty and others[27] evaluated the effectiveness of Lactobacillus strains in preventing the growth of E. faecalis and observed that Lactobacilli had an inhibitory effect on the growth of E. faecalis. Hasslöf and others[28]investigated the ability of different lactobacilli strains, to inhibit growth of C.albicans in vitro. Their result showed that Rhamnosus displayed a slight inhibition of C.albicans. Among previous human clinical studies, Lactobacillus Rhamnosus[29] could inhibit the oral cariogenic bacteria. In another study it was found that Rhamnosus could reduce the prevalence of yeast in elder persons.[30]Similar study was done by Kraft-Bodi and others[31] found a statistically significant reduction in the prevalence of high Candida counts in the probiotic group containing Lactobacillus strains but not in the placebo group. Contrary to this study, Ahola and others[32] observed no significant difference between effects of probiotic and those of control cheese on salivary Candida counts. Denkova and others[29] in their study observed lactobacilli strains to restrain the growth of C.albicans and by the 72 hour of co-cultivation the pathogen retained high concentration of viable cells.

In our study Group 4 (5.25% NaOCl) showed highest zone of inhibition than Group 1, 2, 3 and 5 against E faecalis and C albicans.

This could be due to hypochlorous acid released from NaOCl which is responsible for bacteria inactivation. Hypochlorous acid disrupts oxidative phosphorylation and other membrane-associated activities as well as DNA synthesis.[11,12] Vienna and others[33] showed that resistant microorganism, C Albicans, was killed in vitro in 15 s by 5.25% NaOCl. Siqueira and others[9] using E faecalis infected root canals demonstrated the superior antibacterial affect against root canal bacteria by Sodium hypochlorite in comparison with physiological saline.

V. CONCLUSION

Within the limitation of this study, it can be concluded that:

I. All probiotic strains used were able to inhibit growth of E. faecalis and C Albicans at both time periods.
II. Against E. faecalis and C. Albicans, 5.25 % NaOCl showed significantly higher zone of inhibition followed by all probiotic strains at both time periods.
III. There was insignificant difference in zone of inhibition at 48 hours and 1 week.

This in vitro study demonstrated that probiotics show a potential in root canal therapy, but further in vitro and in vivo studies are needed to determine its full potential against different endodontic infections.

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Table 1 shows intergroup comparison of mean zone of inhition (ZOI) against C.albicans and E.faecalis at 48 hours and 1 week

Antimicrobial Agents	C.albicans		E.faecalis	
	ZOI (mm) At 48 hours	ZOI(mm) At 1 week	ZOI (mm) At 48 hours	ZOI (mm) At 1 week
Group1:Lactobacillus Acidophilus	5.6867^{A}	6.2533^{B}	5.1433^{C}	6.6700^{D}
Group2: Lactobacillus Casei	2.4333 ^{A}	2.2233 ^{B}	3.8433 ^{C}	4.9667 ^{D}
Group3: Lactobacillus. Rhamnosus	1.0067 ^{A}	1.0333 ^{B}	1.1633 ^{C}	3.3667 ^{D}
Group4: 5.25% Sodium Hypochlorite	9.0267 ^{A}	9.1367 ^{B}	11.5267 ^{C}	10.1467 ^{D}
Group5: Normal Saline	0 ^{A}	0 ^{B}	0 ^{C}	0 ^{D}

Values with same letters indicate statistically significant differences

Table 2 shows intergroup comparison of mean number of colony counts against C.albicans at 72 hours.

Organisms	GROUP 1	GROUP 2	GROUP 3	GROUP 4	Control Avg cfu/ml

Candida Albicans	4×10^{4}	1×10^{3}	1.5×10^{3}	3.8×10^{3}	7.0×10^{5}
Log $_{10}$ Test Avg	4.60^{A}	3.0^{A}	3.17^{A}	4.57^{A}	5.84^{A}

Values with same letters indicate statistically significant differences

Table 3 shows intergroup comparison of mean number of colony counts against E.faecalis at 72 hours

Organisms	**GROUP 1**	**GROUP 2**	**GROUP 3**	**GROUP 4**	**Control Avg cfu/ml**
E.faecalis	10^{-1}	10^{-2}	10^{-3}	4.4×10^{3}	3.5×10^{5}
Log $_{10}$ Test Avg	-1^{A}	-2^{A}	-3^{A}	3.64^{A}	5.54^{A}

Values with same letters indicate statistically significant differences

Legends

Table 1 shows intergroup comparison of mean zone of inhition (ZOI) against C.albicans and E.faecalis at 48 hours and 1 week
Table 2 shows intergroup comparison of mean number of colony counts against C.albicans at 72 hours.
Table 3 shows intergroup comparison of mean number of colony counts against E.faecalis at 72 hours
Figure 1: E.faecalis on blood agar plate (1 a), ZOI's for E. faecalis at 24 hrs (1 b), ZOI's for E. faecalis at72 hrs (1 c)
Figure 2: C.albicans on blood agar plate (2 a), ZOI's for C.albicans at 24 hrs (2 b), ZOI's for C.albicans at72 hrs (2 c)