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A Promising Probiotic Irrigant: An *In Vitro* Study

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Abstract

AIM: The present study aimed to investigate the inhibitory effect of *Lactobacillus rhamnosus* (B-445) as a probiotics irrigant on the growth of *Enterococcus faecalis*.

METHODS: Forty-two extracted single human canal anterior teeth were prepared with rotary instrumentation and sterilised. Teeth were divided into 3 groups according to the type of irrigant, N = 14. Three experimental groups were inoculated with *E. faecalis* and cultured for 21 days before use; Group 1 was 2.5% NaOCl (positive control), Group 2 was saline (negative control), Group 3 was the experimental probiotic irrigant. Paper point sampling of the canals of each group was obtained before irrigation (S1), immediately after irrigation (S2) and after 24 hours (post irrigation samples) (S3) to determine remaining colony forming units for *E. faecalis*. Also, Colony counts for *L. rhamnosus* in Group 3 after immediate irrigation, as well as 24 hours post irrigation, was performed to determine the survival profile of these bacteria in infected root canal with *E. faecalis.*

RESULTS: The NaOCl irrigant group had the lowest mean value of (log 10 CFU/mL) of *E. faecalis* after immediate irrigation and after 24 hrs post irrigation followed by the probiotic group, while the highest mean value was the saline group ($P \leq 0.001$). The survival profile for *L. rhamnosus* in Group 3 after immediate irrigation and post-irrigation were slightly higher than for *E. faecalis* ($P \leq 0.001$).

CONCLUSION: *Lactobacillus rhamnosus* which revealed a potential inhibitory effect on the growth of *Enterococcus faecalis*, could be used as a new natural, safe probiotic irrigant agent.

Introduction

In Endodontics, success depends on chemomechanical removal of bacteria, bacterial endotoxin and debris from the root canal system (Sjögren et al., [1] 1997; Katebzadeh et al., [2] 2000). *Enterococcus faecalis* (*E. faecalis*) is an anaerobic, facultative microorganism that is highly resistant to conventional chemomechanical preparation and usually found in failing root canal cases [3]. *E. faecalis* is a part of the human flora and appears to be highly resistant to the medicaments used in treatment [4]. Also, *E. faecalis* can form a surface attached microbial community known as a biofilm. This allows it to be protected from host defences as well as systemic treatment.

Different methods of combatting *E. faecalis* were explored through the use of different irrigants [5]. Sodium hypochlorite has remained a popular root canal irrigant because of its ability to dissolve necrotic tissue and organic remnants and its antimicrobial activity. On the other hand, sodium hypochlorite has certain adverse effects such as ineffectiveness against some microorganisms when used at low concentration, corrosion of endodontic instruments and lack of differentiation between necrotic and vital tissues when in contact with apical and periapical tissues [6]. “Out of the box” treatments were evaluated for use against *E. faecalis* such as the use of passion fruit juice as an endodontic irrigant as well as the use of Photomedicine and laser surgery [7]. In endodontics because of the cytotoxic reactions of irrigants used and their inability to eliminate bacteria from dentinal tubules, the trend of recent medicine attends to use biologic medication.

Probiotics have been shown to promote health in the intestines and the oral cavity through the consumption of certain yoghurts and lozenges. The mechanisms of action of these probiotics include the productions of bacteriocin-like inhibitory substances and the altering of the local pH, competing for nutrients, forming physical barriers and stimulating the immune response [8]. The World Health Organization recognises probiotics as a significant avenue of health preservation if current antibiotics become useless due to the development of bacterial resistance. The purpose of this study was shifting the established paradigm of endodontic treatment from eliminating all bacteria from the canal system to focusing on the elimination of the problematic bacteria through introducing probiotics.

Therefore, this study aimed to explore the probiotics as new irrigant agent that would probably have an inhibitory effect against the growth of Enterococcus faecalis.

Material and Methods

Forty-two freshly extracted single-rooted human teeth with fully formed apices were collected for this study. All teeth were examined under a stereomicroscope (Technical, Germany) to exclude teeth with any existing defects or cracks. Teeth were thoroughly washed under running water and immersed in 5.25% Sodium Hypochlorite (Clorox, Egyptian company for household products, Egypt) for 30 minutes to remove soft tissues on the root surface. Teeth were gently cleaned with ultrasonic scaler to remove any remaining soft tissues or calculus. The teeth were stored in saline solution until instrumentation. Before canal instrumentation, the crown of each tooth was sectioned at cementoenamel junction using a sectional disc (Toolouip, Germany) mounted in a low-speed handpiece (NSK, Japan) under water coolant. This was done for ease of manipulation and to standardise the tooth length at 15 mm.

A size 15 k-type file (Mani, Japan) was used to assure patency of the canal and the apical foramen. The working length was established by subtracting 1 mm from the length obtained when the file tip just appeared at the apical foramen. Cleaning and shaping were performed by using rotary Ni-Ti Protaper system that was driven by an endodontic handpiece of 1:16 gear reduction and powered by X-Smart electric motor (Speed 400 rpm and torque setting 5 Ncm, Dentsply, Japan). All canals were enlarged to the same file size F5. During instrumentation, irrigation was performed with 3 ml of 5.25% sodium hypochlorite after each file. After completion of the instrumentation, the smear layer was removed with 3 ml 17% ethylene-diamine-tetra-acetic acid solution (EDTA-Q.A-Dent, Egypt) by using a disposable plastic syringe. This was followed by irrigation with distilled water. Teeth were then autoclaved at 121°C for 30 minutes. After that, the apical foramen of each tooth was sealed by using Putty silicone for impression, and the root surface was sealed with varnish after which the samples were handled under strict aseptic measures.

Lactobacillus rhamnosus (*L. rhamnosus* B-445) was selected as an example of probiotic species and provided in lyophilised form by the Northern Regional Research Laboratory, Illinois, USA. Bacterial activation was carried out through inoculation of *L. rhamnosus* in De Man, Rogosa, Sharpe (MRS) broth medium (Oxide, Basingstoke, Uk) followed by anaerobic incubation (BBL Gas Pak, Becton Dickinson, Cockeysville, MD, USA) at 37°C for 48 hrs. The experimental probiotic irrigant was formulated through inoculation 5 ml of *L. rhamnosus* ($2x10^8$ CFU/ml) in 10 ml of sterile distal water.

A Total of Forty-two samples were randomly assigned to three main groups according to the type of irrigant was applied (N = 14); Group1 was 2.5% NaOCl (positive control), Group2 was saline (negative control), Group3 was the experimental probiotic irrigant.

The three groups were infected by using a 24-hours pure culture suspension of *Enterococcus faecalis* (*E. faecalis*; ATCC 19434) in Brain heart infusion broth medium (BHI broth medium; 53286 Sigma-Aldrich, USA). In each previously sterilised samples 1 x 10^{-8} CFU/mL suspension of *E. faecalis* (determined by serial dilution and plating) was inoculated in root canals and incubated at 37°C and 95% humidity for 21 days. The BHI broth was removed from the canal by gentle aspiration and renewed every 48 hours.

At the end of 21 days infection with *E. faecalis*, sterile paper point pre-irrigation sample was taken from each specimen. The sterile paper points were placed in the canals to the apical foramen and were moved circumferentially along the walls of the canal. Each paper point was left for 1 minute in the canal to collect the pre-irrigation samples from the canals of all teeth. Paper points were placed in airtight sterile vials containing 5 ml of nutrient broth for transportation to the microbiological laboratory for culturing procedure. After that, each group was irrigated immediately with 5 mL of its specific irrigant, and all irrigants were delivered by using a side-vented 30-gauge needle (Max-I-Probe; Dentsply). The immediate paper point irrigation sample was obtained from each root canal of each tested groups. Again the experiment was repeated after 24 hours irrigation of root canals (post irrigation samples) and storage in an incubator at 37°C.

In colony counting method, each pre-irrigation, immediate, post irrigation paper points samples were placed in airtight sterile vials containing 5 ml of saline and vortexed at the highest speed for 3

consecutive intervals of 15 seconds each. This was followed by serial dilutions 1:10, 1:100, 1:1000 and 1:10000. A volume of 50 µl was taken from each dilution using the automatic micropipette and was plated on BHI agar in triplicate. All plates were incubated at 37°C and 95% humidity for 24 hours, after which the colonies for *E. faecalis* on BHI agar plates were enumerated, and the most countable plates from pre-irrigation, immediate, post irrigation paper point's samples of each group were selected. If any sample was too numerous to count, it was diluted, plated, and counted.

Colony counts for *E. faecalis* and *L. rhamnosus* in Group 3 after immediate irrigation as well as 24 hours post irrigation was performed to determine the survival profile of these bacteria. Colony count method was carried out as mentioned above through serial dilutions followed by a volume of 50 µl was taken from each dilution and plated on BHI agar in triplicate. Also, the duplicated paper point samples were plated on MRS agar in triplicate. The experiment was repeated after 24hrs post irrigation. All plates were incubated at 37°C and 95% humidity for 24 hours, after which both the colonies for *E. faecalis* on BHI agar plates and *L. rhamnosus* on MRS agar plates were enumerated and the most countable plates for each microorganism were selected.

Data presented as means and standard deviation (SD) values. One-way repeated measures ANOVA used to compare between the follow-up within each Irrigant solution. One Way ANOVA used to compare different irrigant solutions within each time.

For the Survival profile of microorganisms (Log10 CFU/mL); independent t-test used to compare between different type of bacteria, Dependent t-test used to compare between follow-up periods for each irrigant. The significance level was set at P ≤ 0.05 and was performed with IBM® SPSS® (SPSS Inc., IBM Corporation, NY, USA) Statistics Version 24 for Windows.

Results

Regarding the colony counting method, the results showed that there was a statistically significant difference in the mean (log10 CFU/mL) of *E. faecalis* between the three irrigants groups immediately and after 24 hrs post irrigation (P ≤ 0.001). While there was no statistically significant difference in the mean (Log10 CFU/mL) of *E. faecalis* between tested groups pre-irrigation (P = 0.001).

Table 1: Mean and SD for the Log10 CFU of (*E. faecalis*) for different irrigants

		Groups						p-value
		Saline		NaOCl		Probiotic		
		Mean	SD	Mean	SD	Mean	SD	
Log10 CFU (E. faecalis)	Pre-irrigation	7.04^{Ab}	0.03	7.20^{Aa}	0.07	7.09^{Ab}	0.09	0.001*
	Immediately	4.95^{Ba}	0.11	0.00^{Cc}	0.00	4.40^{Bb}	0.10	≤ 0.001*
	After 24 hours	6.89^{Aa}	0.32	2.20^{Bc}	2.01	4.04^{Bb}	0.16	≤ 0.001*
p-value		≤ 0.001*		≤ 0.001*		≤ 0.001*		

Means with same Capital Superscript within each Column are not significantly different at p=0.05; Means with same Small Superscript within each Row are not significantly different at p = 0.05; NS = Non-significant; * = Significant.

The previous results revealed that the NaOCl irrigant group had the lowest mean value of (log10 CFU/mL) of *E. faecalis* after immediate irrigation (0.00) and after 24 hrs post irrigation (2.20 ± 2.01) followed by the probiotic group with mean value (4.95 ± 0.11) for immediate irrigation and (4.40 ± 0.10) for post irrigation, while the highest mean value immediately and after 24 hrs post irrigation was the saline group (4.95 ± 0.11, 6.89 ± 0.32) respectively (Table 1, Figure 1).

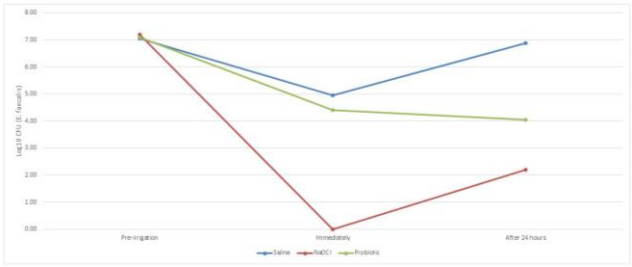

Figure 1: Line chart showing the mean Log10 CFU (E. faecalis) for follow-up periods for each group

Concerning the survival profile for both bacterial growth *E. faecalis* and *L. rhamnosus* in Group 3, the results showed that the mean colony count (Log10 CFU/mL) for *L. rhamnosus* after immediate irrigation and post-irrigation (6.41 ± 0.06, 4.77 ± 0.44) was slightly higher than for *E. faecalis* (4.40 ± 0.10, 4.04 ± 0.16 respectively) (Table 2, Figure 2).

Table 2: Mean and SD for the Survival profile of microorganisms (Log10 CFU/mL)

		Bacterial				p-value
		E. faecalis		Lactobacillus Rhamnosus (L.rhamnosus)		
		Mean	SD	Mean	SD	
Survival profile of microorganisms (Log10 CFU/mL)	Immediately	4.40	0.10	6.41	0.06	≤ 0.001*
	After 24 hours	4.04	0.16	4.77	0.44	≤ 0.001*
p-value		≤ 0.001*		≤ 0.001*		

*NS = Non-significant; * = Significant.*

Discussion

As *Enterococcus faecalis* (*E. faecalis*) became more and more commonly isolated in failing root canal cases, it became the focus of interest in the medical and dental communities. Hence, *E. faecalis*

was selected in our due to its ability to form a surface attached microbial community known as a biofilm. This allows it to be protected from host defences as well as systemic treatment [9], [10]. Additionally, It is the most common bacterial species found in persistent infections due to its relatively small cell diameter [11], [12]. The present study was designed to prepare a standardised tooth length of 15 mm, and the root canals were enlarged to F5 Protaper at the working length as this could attribute to create an adequate environment for bacterial growth.

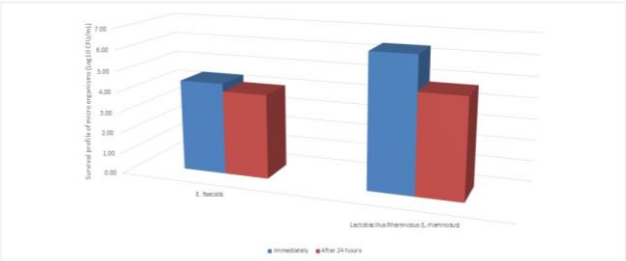

Figure 2: Histogram showing the mean Survival profile of microorganisms (Log10 CFU/mL)

Based on previous studies, the counting of CFUs for bacterial growth that was expressed in log CFU/mL could be used in the present study to evaluate the effects of the tested irrigants on *E. faecalis* growth [13], [14], [15], [16]. Several endodontic irrigants have been used for decontamination, but the most common was sodium hypochlorite (NaOCL) for its antimicrobial activity [17], [18], [19] and its ability of dissolution of organic tissues [20], [21]. However, due to its high toxicity [22], new irrigants were introduced. Therefore, this study was trying to explore a potential new way of treating endodontic infections which would probably be as effective or more and at the same time less irritating to the tissues than sodium hypochlorite. The use of probiotics has not yet been evaluated for use against *E. faecalis*. So, the present study was proposed to test known probiotics as an experimental irrigant in the presence of the established post-endodontic treatment disease bacteria *E. faecalis* and observe what the effect of the probiotics would have against *E. faecalis.*

Regarding colony counting method, the results showed that *E. faecalis* colonies statistically significant decrease from (7.09 log CFU/mL) pre-irrigation to (4.04 log CFU/mL) after 24 hrs post irrigation with probiotic irrigant. The latter result was supported by [23] who documented that killing of pathogens through probiotic could be due to one of the mechanisms of probiotic action such as; the production of Bacteriocin-like inhibitory substance (BLIS) and acids/peroxides along with altering the local environments pH. However, (BLIS) is used to describe bacterial products that have inhibitory effects.

For the results that concerning the survival profile for both bacterial growth *E. faecalis* and *L. rhamnosus* in Group 3, it revealed that the mean colony count for *L. rhamnosus* after immediate irrigation and post-irrigation was statistically significantly higher than for *E.faecalis* ($P \leq 0.001$). A more sophisticated method of observing the effects of the probiotic agents against *E. faecalis* could be conducted. The method of “Deferred Antagonism” which was demonstrated by [24] could explain the latter result that been used to evaluate the antibacterial properties of the normal flora of the nasopharynx. This deferred antagonism test which previously mentioned could be used to evaluate our hypothesis presented in a more controlled fashion as probiotic might compete for *E. faecalis* on nutrition and adhesion site which contribute to decreasing the survival profile of *E. faecalis* as found in the latter result of the present study. *E. faecalis* appears to be highly resistant to the medicaments used in the treatment and is one of the few microorganisms shown in vitro to be resistant to calcium hydroxide, due to its proton pump [4]. It is also able to survive as a single organism without the support of other bacteria. The fact that *E. faecalis* is not normally present or is present in very low numbers in untreated root canal cases implies that it can enter the canal, survive the antibacterial treatment and then persist after obturation [25]. Therefore, this deferred antagonism test which previously mentioned could be used to evaluate our hypothesis presented in a more controlled fashion as probiotic might compete for *E. faecalis* on nutrition and adhesion site which contribute to decreasing the survival profile of *E. faecalis* as found in the latter result of the present study.

In conclusion, within the limitation of this study; *Lactobacillus rhamnosus* which revealed a potential inhibitory effect on the growth of *Enterococcus faecalis*, could be used as a new natural, safe probiotic irrigant agent. Further studies using other known probiotics should be considered.

References

1. Sjögren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J. 1997; 30(5):297–306. https://doi.org/10.1111/j.1365-2591.1997.tb00714.x PMid:9477818

2. Katebzadeh N, Sigurdsson a, Trope M. Radiographic evaluation of periapical healing after obturation of infected root canals: an in vivo study. Int Endod J. 2000; 33(1):60–6. https://doi.org/10.1046/j.1365-2591.2000.00301.x PMid:11307475

3. Pinheiro ET, Gomes BPFA, Ferraz CCR, Sousa ELR, Teixeira FB, Souza-Filho FJ. Microorganisms from canals of root-filled teeth with periapical lesions. Int Endod J. 2003; 36(1):1–11. https://doi.org/10.1046/j.1365-2591.2003.00603.x PMid:12656508

4. Bystrom a, Sundqvist G. The antibacterial action of sodium

hypochlorite and EDTA in 60 cases of endodontic therapy. Int Endod J. 1985; 18(1):35–40. https://doi.org/10.1111/j.1365-2591.1985.tb00416.x PMid:3922900

5. Shingare P, Chaugule V. Comparative evaluation of antimicrobial activity of miswak, propolis, sodium hypochlorite and saline as root canal irrigants by microbial culturing and quantification in chronically exposed primary teeth. GERMS. 2011; 1(1):12–21. https://doi.org/10.11599/germs.2012.1004 PMid:24432254 PMCid:PMC3882825

6. Seltzer S, Farber PA. Microbiologic factors in endodontology. Oral Surgery, Oral Med Oral Pathol. 1994; 78(5):634–45. https://doi.org/10.1016/0030-4220(94)90178-3

7. Jayahari NK umar, Niranjan NT, Kanaparthy A. The efficacy of passion fruit juice as an endodontic irrigant compared with sodium hypochlorite solution: an in vitro study. J Investig Clin Dent. 2014; 5(2):154–60. https://doi.org/10.1111/jicd.12023 PMid:23355285

8. Krasse P, Carlsson B, Dahl C, Paulsson A, Nilsson Å, Sinkiewicz G. Decreased gum bleeding and reduced gingivitis by the probiotic Lactobacillus reuteri. Swed Dent J. 2006; 30(2):55–60. PMid:16878680

9. Chávez de Paz LE, Bergenholtz G, Svensäter G. The Effects of Antimicrobials on Endodontic Biofilm Bacteria. J Endod. 2010; 36(1):70–7. https://doi.org/10.1016/j.joen.2009.09.017 PMid:20003938

10. George S, Kishen A, Song KP. The role of environmental changes on monospecies biofilm formation on root canal wall by Enterococcus faecalis. J Endod. 2005; 31(12):867–72. https://doi.org/10.1097/01.don.0000164855.98346.fc PMid:16306820

11. Kayaoglu G, Ørstavik D. Virulence factors of Enterococcus faecalis: relationship to endodontic disease. Crit Rev Oral Biol Med. 2004; 15(5):308–20. https://doi.org/10.1177/154411130401500506 PMid:15470268

12. Portenier I, Waltimo TMT, Haapasalo M. Enterococcus faecalis– the root canal survivor and 'star' in post-treatment disease. Endod Top. 2003; 6(1):135–59. https://doi.org/10.1111/j.1601-1546.2003.00040.x

13. Gründling GL, Zechin JG, Jardim WM, De Oliveira SD, De Figueiredo JAP. Effect of ultrasonics on enterococcus faecalis biofilm in a bovine tooth model. J Endod. 2011; 37(8):1128–33. https://doi.org/10.1016/j.joen.2011.05.006 PMid:21763907

14. Menezes MM, Valera MC, Jorge AOC, Koga-Ito CY, Camargo CHR, Mancini MNG. In vitro evaluation of the effectiveness of irrigants and intracanal medicaments on microorganisms within root canals. Int Endod J. 2004; 37(5):311–9. https://doi.org/10.1111/j.0143-2885.2004.00799.x PMid:15086752

15. Evans M, Baumgartner J, Khemaleelakul S, Xia T. Efficacy of Calcium Hydroxide: Chlorhexidine Paste as an Intracanal Medication in Bovine Dentin. J Endod. 2003; 29(5):338–9. https://doi.org/10.1097/00004770-200305000-00005 PMid:12775006

16. Peters LB, Wesselink PR, Moorer WR. The fate and the role of bacteria left in root dentinal tubules. Int Endod J. 1995; 28(2):95–9. https://doi.org/10.1111/j.1365-2591.1995.tb00166.x PMid:7665208

17. Siqueira JF. Antibacterial effects of endodontic irrigants on black-pigmented gram-negative anaerobes and facultative bacteria. J Endod. 1998; 24(6):414–6. https://doi.org/10.1016/S0099-2399(98)80023-X

18. Niazi SA, Clark D, Do T, Gilbert SC, Foschi F, Mannocci F, et al. The effectiveness of enzymic irrigation in removing a nutrient-stressed endodontic multispecies biofilm. Int Endod J. 2014; 47(8):756–68. https://doi.org/10.1111/iej.12214 PMid:24246147

19. Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. Effect of long-term exposure to endodontic disinfecting solutions on young and old Enterococcus faecalis biofilms in dentin canals. J Endod. 2014; 40(4):509–14. https://doi.org/10.1016/j.joen.2013.11.026 PMid:24666901

20. Okino LA, Siqueira EL, Santos M, Bombana AC, Figueiredo JAP. Dissolution of pulp tissue by aqueous solution of chlorhexidine digluconate and chlorhexidine digluconate gel. Int Endod J. 2004; 37(1):38–41. https://doi.org/10.1111/j.1365-2591.2004.00749.x PMid:14718055

21. Dutta A, Saunders WP. Comparative evaluation of calcium hypochlorite and sodium hypochlorite on soft-tissue dissolution. J Endod. 2012; 38(10):1395–8. https://doi.org/10.1016/j.joen.2012.06.020 PMid:22980186

22. Tanomaru Filho M, Leonardo MR, Silva LAB, Aníbal FF, Faccioli LH. Inflammatory response to different endodontic irrigating solutions. Int Endod J. 2002; 35(9):735–9. https://doi.org/10.1046/j.1365-2591.2002.00544.x PMid:12449023

23. Gupta G. Probiotics and periodontal health. J Med Life. 2011; 4(4):387–94. PMid:22514571 PMCid:PMC3227153

24. Walls T, Power D, Tagg J. Bacteriocin-like inhibitory substance (BLIS) production by the normal flora of the nasopharynx: Potential to protect against otitis media? J Med Microbiol. 2003; 52(9):829–33. https://doi.org/10.1099/jmm.0.05259-0 PMid:12909662

25. Sundqvist G. Associations between microbial species in dental root canal infections. Oral Microbiol Immunol. 1992; 7(5):257–62. https://doi.org/10.1111/j.1399-302X.1992.tb00584.x PMid:1494447