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EVALUATION AND COMPARISON OF THE ANTIMICROBIAL EFFICACY OF CALCIUM HYDROXIDE & PROBIOTIC STRAIN (LACTOBACILLUS RHAMNOSUS) AGAINST C. ALBICANS

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ABSTRACT

Aim: to evaluate and compare the effect of probiotic strain (Lactobacillus Rhamnosus) and calcium hydroxide against Candida albicans (C. albicans).

Materials and methods: 40 human single-rooted teeth were decoronated then instrumented using PLEX-V rotational file system up to PLEX-V 40.04. After being infected with Candida albicans the samples were divided into four equal experimental groups at random based on the type of intracanal medicament used. The roots were cultured for seven days after being arranged vertically along their long axis in a wax block. Using H-files, shaved dentin chips were gathered for bacterial culture. To measure the antibacterial activity, bacterial colony-forming units per milliliter (CFUs/ml) were used.

Results: The negative control group had the greatest count, followed by calcium hydroxide, then the probiotic group, while the lowest count was found in calcium hydroxide+ probiotic group.

Conclusion: Upon incubation for seven days, the combination proved to be the most successful in reducing the bacterial load of C. albicans.

KEYWORDS: probiotics, Lactobacillus Rhamnosus (L. Rhamnosus), Intracanal medicament.

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INTRODUCTION

Root canal therapy helps to avoid recurrent infections by removing pathogens and their byproducts from root canals. However, germs can survive in empty channels, and not all bacterial species are eradicated. A proper root canal dressing with long-lasting antibacterial activity, strong fungicidal and germicidal properties, and inhibits the growth of bacteria in between treatments is used.

Chemo-mechanical root canal preparation is often combined with intracanal medications to effectively manage the elimination of microbial contamination from the pulp space. The most popular intracanal medication (ICM) for treating apical periodontitis is calcium hydroxide (CH). The release of hydroxyl ions causes its high alkalinity, which destroys the protein structure, DNA, and bacterial cell membrane. [1]

By competing with pathogenic bacteria for resources, probiotics can inhibit their growth and reduce the formation of toxic biofilms. They are typically bacteria, which are beneficial microorganisms that are like the microbes found in human bodies. Yoghurt and fermented foods are natural sources of probiotics. They are employed in numerous therapeutic and medical domains. [2]

Lactobacillus strains have become popular probiotics with the potential to treat and prevent infections. Because of their "Generally Recognized as Safe" (GRAS) classification and potent antagonistic actions against a variety of human pathogens. [3]

The normal probiotic strain found in the oral cavities of people without periodontitis or dental cavities is L. Rhamnosus, which is also known to have activity against a wide range of bacteria that cause periodontal disease. [4,5] This study shed light on the effect of L. Rhamnosus against C. albicans in comparison with CH and combination of CH with probiotic when used as intra-canal medicament during endodontic treatment. A proposed null hypothesis suggests that there is no difference in the effectiveness of using probiotics, CH, or a combination of both as intracanal medicaments during endodontic treatment.

MATERIALS AND METHODS

Selection of samples

An ethical clearance obtained for the proposal of the research from the ethics and postgraduate committee of the faculty of Dentistry, Minia University in their session (Meeting no 98, Date:25/7/2023, Approved no 762) before carrying out the study from the department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Minia University, forty human teeth with a single root that had been extracted for periodontal or orthodontic reasons were gathered. Preoperative radiographs in both buccolingual and mesiodistal directions were taken. Roots with carious lesions, resorption, calcification and fracture were rejected.

Sample preparation:

Following two minutes of surface disinfection and soft tissue dissolution in NaOCl 5.25% (NaOCl; Clorox, HC Egyptian company, Cairo, Egypt), the external root surfaces of all collected teeth were cleaned with a curette to remove calculus and periodontal tissues. To avoid dehydration, the teeth were then preserved in saline. Crowns were cut off until each root measured 15 mm.

Sterile ISO K-files of size #15 were inserted to evaluate each canal's patency. (Micro Mega SA, Besancon, France) into the apical foramen and pushing back until the file flushed with the visible apical foramen, up to the PLEX V 40.04 rotary file (Orodeka, Italy), all teeth were mechanically prepared in compliance with the manufacturer's instructions with the aid of the PLEX V Rotary File System and COXO Wireless Endo-motor (COXO Medical Instrument Co., Ltd). at speed 300 rpm and 1.5N.cm as a torque according to the instructions of manufacture. [6]

Three milliliters of 2.5% sodium hypochlorite (NaOCl) and one milliliters of 17% ethylene diamine tetraacetic acid (EDTA Prevest Denpro Limited/REF 50002/India) were used to irrigate the samples in order to remove both organic and inorganic debris. To get rid of any residual previous irrigants, 5 milliliters of distilled water were used to irrigate each sample using 30-gauge needle (Fanta Dental. China) then samples were autoclaved at 121 °C for 20 min using steam air autoclave (Rundeer Control Equipment Co, Ltd) [7]

Preparation of microbial culture:

C.albicans strains were obtained from Reference laboratory of Egyptian University Hospitals -Cairo. They were cultivated in Yeast Extract—Peptone-Dextrose Broth (YPD) at 30 °C until they reached the log phase. The microbial suspension was adjusted to optical density of 0.1 at 600 nm being inoculated into the root canals. [8]

Probiotics culture and supernatant preparation

The strain of Lactobacilli Probiotics (L.Rhamnosus ATCC 7469) was obtained from Central food safety lab (CFSL), Faculty of Agriculture, Ain Shams University, Cairo, Egypt and grown on De Man, Rogosa, and Sharpe (MRS) broth in an aerobic environment for 48 hours at 37 °C. MRS broth in a 100 ml flask was utilized to cultivate the pure isolate of Lactobacilli species suspension. It was then kept for incubation at 37 °C for 72 hours. The cell-free supernatant (CFS) was obtained by centrifuging the culture at 10,000 rpm for ten minutes, sterilizing it with a 0.45-micron syringe filter, and then lyophilizing it in a vacuum freezer dryer. (Faculty of Science Minia university) Lyophilized CFS was combined with sterile water to create a Probiotic Supernatant solution. [9]

Sample Classification:

All samples (n = 40) were randomly divided into 4 groups according to medication.

• Group A (n= 10): No medication (negative control)

• Group B (n= 10): Probiotic strain (L. Rhamnosus)

• Group C (n= 10): Calcium hydroxide.

• Group D (n= 10): CH + L. Rhamnosus.

The root samples' infection:

The root canals were injected with a 30 µl log phase culture of C. albicans using an insulin syringe, and Teflon was used to plug the canal orifices. To produce mature biofilms, the root samples of Candida albicans were cultivated for seven days. Each procedure was carried out in the Faculty of Pharmacy at Minia University.

Intra-canal medication:

Following the period of contamination, 3 ml of sterile saline were used to irrigate each specimen, and sterile paper points #40 (GAPADENT CO., LTD., TianJin City, P.R. China) were used to dry them

- **Group A:** No medication.
- **Group B:** An insulin syringe was used to inject a probiotic supernatant solution into each root canal. The entire volume was applied to ensure the canal was filled.
- **Group C:** Metapaste (MetaPaste, MetaBiomed, Seoul, Korea) was placed by the tip of the ready-made injectable paste to make sure that the medicament filled all the canal and got in contact with all walls.
- **Group D:** CH powder was mixed with Probiotic Supernatant solution in a ratio of 1.5:1 (weight/volume) to obtain a paste-like consistency. A disposable tip of metapaste was fitted onto a disposable syringe. The mix was loaded in the syringe, which was then tapped on a solid surface till the bubbles entrapped were removed. [10]

Methods of evaluation:

After sealing the canal orifices with Teflon and placing the specimens in dental modelling wax. Samples were cultured for seven days at 37 C.

The Teflon seal was taken off following the incubation time. PLEX V X4 was utilized at 300 RPM and 2 N.cm torque to remove the medication. After irrigating the root canals with sterile saline solution, sterile paper points # 40 were used to dry them. Sterile #40 H-files are used to harvest dentin from the root canals of every specimen in each group, along the working length of the root canal.

To count the colonies of C. albicans, 20 µl of this solution was plated on (YPD) agar for 48 hours, after each file was placed in a sterile Eppendorf test tube with 1 ml of sterile saline and vortexed for 30 seconds.

Colony forming units (CFU) were used to count and record growing colonies. [II] every outcome was gathered, totaled, and subject to statistical analysis

Statistical analysis:

Numerical data were presented as mean and standard deviation values. Normality and variance homogeneity assumptions were verified by viewing the data distribution and using Shapiro-Wilk's and Levene's tests, respectively. The data were found to be non-parametric and were analyzed using Kruskal-Wallis's test, followed by Dunn's post hoc test with Bonferroni correction. Correlation analysis was made using Spearman's rank-order correlation coefficient. The significance level was set at $p<0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.4.1 for Windows.*

RESULTS

Intergroup comparisons and summary statistics for C. albicans count (CFU/ml) *103 are presented in table (1) and figure (1)

Significant differences existed between the various groups ($p<0.001$). The negative control group had the greatest count (10.25±0.26). Afterward, calcium hydroxide (1.80±0.21), then the probiotic group (1.20±0.27), whereas the calcium hydroxide +probiotic group had the lowest count (0.94±0.39).

Post hoc pairwise comparisons showed the negative control to have a significantly higher count than other groups except for calcium hydroxide ($p<0.001$).

In addition, they showed calcium hydroxide to have a significantly higher count than the calcium hydroxide+ probiotic group ($p<0.001$).

TABLE (1) Intergroup comparisons, mean and standard deviation (SD) for Candida Albicans count (CFU/ml) *103

Candida Albicans count (CFU/ml)*10^3 (Mean±SD)				p-value
Negative control	Probiotic	Calcium hydroxide	Calcium hydroxide+ Probiotic	
$10.25±0.26^{A}$	$1.20±0.27^{BC}$	$1.80±0.21^{AB}$	$0.94±0.39^{C}$	<0.001*

Values with different superscripts are significantly different, * significant (p<0.05).

* R Core Team (2024). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

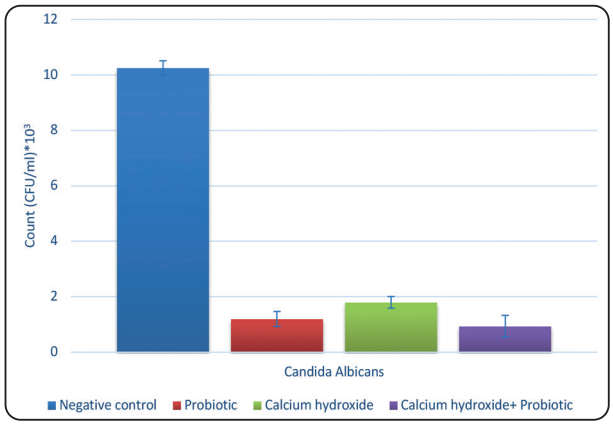

Fig. (1) Bar chart showing mean and standard deviation values of Candida Albicans count (CFU/ml)*103.

DISCUSSION

Despite extensive chemomechanical cleaning and shaping, it may not be possible to completely remove bacterial infections in infected root canals.[12] Because ICMs significantly decrease the pathogen that causes AP, endodontists frequently employ them as a supplement treatment for AP. But according to some research, the bacterial burden in an infected root canal system might have increased or stayed constant.[13]

C.albicans have been associated with persistent post-treatment apical periodontitis. The capacity of C. albicans to transition between the hyphal and blastospore forms is a vital component of its pathogenicity. This enables it to penetrate host tissue and avoid macrophage phagocytosis.[14,15] C. albicans can penetrate deep into dentinal tubules via thigmotropism. Within 48 hours, it can form a biofilm and may lead to persistent infection due to its ability to thrive in various pH levels, highly alkaline environments, and harsh ecological conditions. [8]

Probiotics are described as "live microorganisms that provide a health benefit to the host when taken in adequate quantities"[16] To eliminate harmful microorganisms, they produce bacteriocins, hydrogen peroxide, and lactic acid. Hydrogen peroxide destroys bacteria by disrupting their cell walls, while bacteriocin is a peptide-based toxin that targets bacteria.[17] Probiotics also display competitive behavior for nutrients and space, enhancing their survival capacity in comparison to pathogenic bacteria.[18] A study by Bohora et al[19] showed the antimicrobial effectiveness of commercially available probiotics containing Lactobacillus species against E. faecalis and C. albicans in both biofilm and planktonic state.

Since calcium hydroxide intracanal medication remains the most used endodontic treatment globally, it was employed in this study. According to (Komorowski R 2000) the inclusion of calcium hydroxide in a paste formulation creates a more stable physical barrier, enabling it to stay in the root canal longer and delay recontamination. The authors reported that the antibacterial properties of calcium hydroxide (CH) alone were significantly weaker compared to when it was mixed with chlorhexidine (CHX). [20]

The purpose of this study was to evaluate the antibacterial properties of many intracanal medicaments, including calcium hydroxide paste, probiotic (supernatant of L. rhamnosus), and a combination of calcium hydroxide and probiotic against C. albicans.

Forty single-rooted human teeth with fully formed apices and straight canals were used in this study. The crown of every tooth was separated at the cementoenamel junction and standardized to a length of 16 mm to ensure uniformity among the specimens. [21]

All decoronated teeth were instrumented using the Plex V file system up to a 40/04 file to ensure proper cleaning and facilitate irrigant penetration into the apical third, creating a large reservoir for the irrigant solution[22]. A 30-gauge needle was used to irrigate the canal with 3 mL of 5.25% sodium hypochlorite (NaOCl) and 17% EDTA for one minute to remove both organic and inorganic debris respectively from the dentinal tubules[23]. Lastly, 2 mL of distilled water was applied to rinse

out any leftover traces of the previous irrigants then the samples were autoclaved for 20 minutes at 121°C in a steam-air autoclave to eliminate all microorganisms. [24,8]

C. albicans (ATCC 10231) was cultured in (YPD) broth, a specific medium for the growth of this yeast, at 30°C until it reached the log phase (the period of exponential bacterial growth) [7]

The probiotic strain L. rhamnosus (ATCC 7469) was cultured at 37°C in an aerobic environment for 48 hours in (MRS) broth, a selective medium that provides characteristic colony size and morphology for lactobacilli and other lactic acid bacteria. [25] Cell-free supernatant (CFS) was prepared by centrifuging the culture at 10,000 rpm for ten minutes, followed by sterilization using a 0.45-micron syringe filter and lyophilization in a vacuum freeze dryer. The lyophilized CFS was then reconstituted with sterile water to prepare a probiotic supernatant solution at a concentration of 200 mg/mL [9] as a prior study indicated, the minimum concentration of multi-strain probiotics required to initiate an inhibitory effect against E. faecalis was 50 mg/mL [26]

This study used a 7-day incubation period, as it is the minimum time required for calcium hydroxide (CH) to function effectively as an inter-appointment medication. [27] Saline was selected for medication removal because it has no antibacterial properties and is commonly used to flush out various medicaments. [28] Following the methodology of [29], microbiological sampling was performed by scraping the dentin wall with a #40 Hedstrom file to collect microorganisms, which were then vortexed for 30 seconds to suspend the attached bacteria prior to cultivating on agar plates.

CFUs/mL were considered in the bacterial count as it offers a precise method for detecting live bacteria and assessing the efficacy of intracanal medications. [30] Previous studies have utilized this technique as a reliable quantitative measure of bacterial count. [31,32]

This study's primary goal was to evaluate the effect of L. Rhamnosus, CH and combination of CH with probiotic when used as intra-canal medicament against C. albicans. The results refute the null hypothesis, which states that there is no distinction between using probiotics, CH or both as intra-canal medicament against C. albicans during endodontic treatment.

The results indicated that the use of L. Rhamnosus alone as an (ICM) was significantly more effective than (CH) alone in combating C. albicans. This enhanced antimicrobial effect may be attributed to the probiotics' production of hydrogen peroxide, lactic acid, and bacteriocins. Bacteriocins are antimicrobial peptides that typically exhibit the strongest inhibitory effects against phylogenetically related strains. Hydrogen peroxide has the potential to induce cellular damage, ultimately leading to the death of susceptible microorganisms. [18] The production of acetic acid and lactic acid by probiotics has the capacity to suppress specific infections. The production of these organic compounds through lactose fermentation could act as an important regulatory mechanism. Upon examining the spectrum of organic acids, it was found that lactic acid and acetic acid were the most significant final products of probiotic-associated metabolism [33]

CH directly kills bacteria through the action of hydroxyl ions, while Lipoteichoic acid delivered from L. Rhamnosus, does not have bactericidal properties but instead interferes with biofilm formation and can disrupt preformed biofilms. They demonstrated similar inhibitory effects on biofilms, but their mechanisms of action appear to be different. [34,35]

Bohora [36] resulted that L. rhamnosus exhibited a 90.60% growth reduction against E. faecalis and an 83.79% against C. albicans in a biofilm stage. In contrast [37] investigated reduction in the growth 90.60% of E. faecalis at the biofilm stage when treated by probiotic microorganisms (L. rhamnosus) and 91.80% reduction in growth of C. albicans.

The results of this study revealed that the combination of CH and probiotics led to a more powerful antimicrobial effect against C. albicans compared to using each treatment alone. The combination typically aims to enhance the antimicrobial activity of CH while preserving its biological and mechanical properties. [38]

The findings of the current study align with those of Seifelnasr et al [39], who investigated five commercial probiotic strains against C. albicans and E. faecalis in two phases in vitro. Their study showed that organisms such as B. longum, L. acidophilus, L. rhamnosus, and L. casei were effective at inhibiting the growth of E. faecalis and C. albicans in both their planktonic and biofilm forms.

Utilizing teeth with a single root and a single canal may be one of the study's limitations. ICM may react differently to E. faecalis and C. albicans due to complex pulp anatomy. Additionally, the maximum incubation period for probiotic supernatants was seven days, a state free of bacteria might result from longer incubation times and greater probiotic supernatant concentrations. Furthermore, two types of bacterial pathogens were used in this investigation; however, multispecies are typically the result of RCT failure.

CONCLUSIONS

In single-rooted teeth with a single pulp space, the combination of calcium hydroxide and probiotics was the most effective in reducing the bacterial load of C. albicans after a 7-day incubation period, compared to using either calcium hydroxide or probiotics alone.

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