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Antibacterial Effect of Lactobacillus casei Supernatant and AH Plus Sealer on Enterococcus faecalis

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ABSTRACT

Background and Objective: The growth of residual bacteria within dental root canals is one of the main causes of root canal treatment failure. As a probiotic with antimicrobial properties, Lactobacillus casei has the ability to inhibit pathogenic microorganisms. The aim of this study is to investigate the antibacterial and anti-biofilm effect of Lactobacillus casei probiotic supernatant alone and in combination with AH Plus sealer on Enterococcus faecalis.

Methods: Enterococcus faecalis strain ATCC 29212 and Lactobacillus casei strain ATCC 7469 were used in this experimental laboratory study. Enterococcus faecalis strain was revived in Brain Heart Infusion Broth (BHI BROTH) and Lactobacillus casei strain in De Man, Rogosa and Sharpe (MRS) Broth under anaerobic conditions. Then, Lactobacillus casei supernatant was prepared. Agar diffusion method was used to investigate the antibacterial properties of the supernatant of Lactobacillus casei and sealer separately and in combination with each other. Furthermore, the anti-biofilm effect of these materials was evaluated using the microtiter plate assay.

Findings: According to the results of this study, the mean non-growth halo diameter of Enterococcus faecalis in the presence of Lactobacillus casei supernatant, AH Plus sealer and their combination was 14.66±0.57, 17.16±1.04 and 27.33±1.25 in 24 hours, 14.16±1.04, 15.75±1.08 and 24.66±1.15 in 48 hours and 13.66±1.52, 14.5±1.32 and 24.33±0.76 mm in 72 hours, respectively. The inhibition percentage of Enterococcus faecalis biofilm formation after treatment with AH Plus sealer, Lactobacillus casei supernatant and their combination was 23%, 17% and 33%, respectively.

Conclusion: The results of this study showed that Lactobacillus casei supernatant and AH Plus sealer have antibacterial and anti-biofilm effects on Enterococcus faecalis. Moreover, the combination of Lactobacillus casei supernatant with AH Plus sealer strengthens these effects.

Keywords: *Probiotic*, *Lactobacillus casei*, *Enterococcus faecalis*.

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Introduction

Root canal treatment failure can occur for various reasons, including the remaining bacteria, lack of thorough cleaning and proper sealing of root canals, insufficient coronal sealing, and non-treatment of some canals (1). Enterococcus faecalis is a gram-positive and catalase-negative facultative anaerobic bacterium that is part of the natural flora of the mouth and is found in root canals (2, 3). Even under very harsh conditions, when there are few nutrients and a highly alkaline pH of about 11.5, Enterococcus faecalis may survive. The ability of Enterococcus faecalis to grow in treated canals and as a biofilm on the walls of the root canal without the help of other bacteria makes this infection highly resistant to antimicrobial agents and root canal treatment (4).

Probiotics are living microorganisms that have a positive effect on the host's health when consumed in sufficient quantities. The term "probiotics" encompasses a diverse group of microorganisms (5). Lactobacillus (lactic acid) bacteria and Bifidobacterium are the dominant strains of probiotics (6, 7). The strong antagonistic properties of Lactobacillus against a wide range of human pathogens have made it a promising therapeutic option for the management and prevention of infections (8, 9). Lactobacillus casei can play an important role in maintaining human health due to its antimicrobial properties. This bacterium helps balance the intestinal microbiota and improves digestion and absorption of nutrients by inhibiting the growth of harmful microorganisms. Moreover, its use in the management of infections, especially in the digestive and oral systems, has been recognized as an effective probiotic (10).

Root canal treatment includes cleaning, shaping and 3D filling of the canal space. For the treatment to be successful, it is essential that the canal is completely cleaned and filled. Complete obturation of the root canal after cleaning and shaping prevents the colonization of oral bacteria and the re-contamination of the periapical tissues and the space inside the root (11). Sealers are materials that effectively fill the voids or gaps between the gutta-percha and the dentin walls. These fillers are important because gutta-percha does not naturally adhere to dentin (12). Root canal sealers have antibacterial properties that can help eliminate bacteria inside the canal. All sealers have maximum toxicity and antibacterial activity at the time of mixing, but with the passage of time and the setting process, these properties decrease (13). AH Plus Sealer is an epoxy resin sealer that has excellent mechanical properties, high radiolucency, low polymerization shrinkage, low solubility, and high durability (14). The main components of Paste A in AH Plus Sealer are mainly Bisphenol A epoxy resins. This composition also includes zirconium oxide, silica, iron oxide pigments and calcium tungstate. Paste B consists of several chemical compounds such as tricyclodecane-diamine, dibenzylamine, aminoadamantane, calcium tungstate, silica, zirconium oxide and silicone oil (15).

Agar diffusion test is a common and reliable method to evaluate the antibacterial effect of root sealers. This test shows the ability of sealer to effectively remove bacteria in the direct environment of the root canal (16). However, it should be noted that the results obtained from this method depend to a large extent on factors such as size, shape, molecular weight, diffusion ability of antimicrobial components, volume and concentration of the substance used, incubation time and its level of contact with the culture medium (17).

The present study was conducted to investigate the antibacterial and anti-biofilm activity of Lactobacillus casei probiotic supernatant, independently and in combination with AH Plus sealer, on Enterococcus faecalis under laboratory conditions.

Methods

This experimental laboratory study was conducted after approval by the Ethics Committee of Hamedan University of Medical Sciences with code IR.UMSHA.REC.1402.562.

The standard strain of Enterococcus faecalis ATCC 29212 was obtained from the microbial bank of Babol University of Medical Sciences. Enterococcus faecalis was revived on blood agar (Myrmedia, Iran) and inoculated in BHI (Brain Heart Infusion Broth) broth (Ibresco, Iran). The medium was incubated for 24 hours at 37°C.

The strain of Lactobacillus casei ATCC 7469 was obtained from the Microbial Bank of Iran Scientific-Industrial Research Organization (Karaj, Iran). For reduction, the strains were cultured in MRS (Man, Rogosa and Sharpe) Broth (Qlab, England), and MRS Agar (Qlab, England) under anaerobic conditions. In order to prepare cell culture supernatant from Lactobacillus casei, first a fresh culture was prepared from it in MRS broth medium. Then, the culture medium was centrifuged at 4°C at 10,000 rpm and passed through a 0.22-micron filter to ensure the absence of any microbial cells (18).

This study has been conducted on three main groups including AH Plus sealer, Lactobacillus casei supernatant and combination of AH Plus sealer with Lactobacillus casei supernatant, along with a negative control group including sterile distilled water.

Agar diffusion method was used to evaluate the antimicrobial properties of the materials. A suspension of Enterococcus faecalis with McFarland standard turbidity of 0.5 was prepared and cultured on Mueller Hinton Agar medium (Ibresco, Iran). A well with a diameter of 8 mm was created on each medium plate. Then, from each of the AH Plus (Dentsply DeTrey) sealer materials, the supernatant of Lactobacillus casei and their combination was poured into each of the wells. Then the plates were incubated at 37°C and the diameter of the growth inhibition zone was measured after 24, 48 and 72 hours (19). The experiment was repeated three times and the mean value of the data was used for statistical analysis.

The anti-biofilm activity of AH Plus sealer alone and in combination with the supernatant of Lactobacillus casei on Enterococcus faecalis was evaluated by the microtiter plate method (20). Biofilm was formed by Enterococcus faecalis (1×10^8 CFU/mL) in a 96-well plate for 72 hours in BHI medium containing 2% glucose. Sealer separately and also in combination with Lactobacillus casei supernatant were placed in contact with 2 ml of saline. After biofilm formation, the contents of each well were removed and the wells were washed three times with PBS. 200 microliters of AH Plus sealer, Lactobacillus casei supernatant and their combination were divided equally in the wells and incubated for 24 hours at 37°C. Then the wells were stained with 200 microliters of 0.1% crystal violet at room temperature for 20 minutes. Excess dye was washed off with distilled water. The wells of the microtiter plate were dried at room temperature and the dye bound to the adherent cells was dissolved with 200 µL of 33% acetic acid. In the last stage, the optical density of each well was determined and recorded using an ELISA reader (Bio-Rad, USA) at a wavelength of 590 nm. Saline solution was used for positive control and sterile culture medium was used as negative control. The results of the test were obtained as percentage of biofilm inhibition after treatment with substances according to the following formula.

$[(C-B) - (T-B)]/[(C-B)] \times 100$

C: Mean absorbance (OD) of control, B: Mean absorbance (OD) of negative control well, T: Mean absorbance (OD) of treatment well.

Statistical analyses were performed using GraphPad Prism version 9.5. One-way analysis of variance was used to determine significant differences between groups, and $p<0.05$ was considered statistically significant.

Results

Based on the results of this research, the highest and lowest average diameters of Enterococcus faecalis non-growth halo was observed in the presence of AH Plus sealer and Lactobacillus casei supernatant and Lactobacillus casei supernatant alone respectively (Figure 1 and Table 1). However, no significant difference was observed between the mean diameter of non-growth halo of Enterococcus faecalis in different groups. Also, the results showed that with the increase of incubation time, the diameter of non-growth halo of Enterococcus faecalis decreased, but this decrease was not statistically significant.

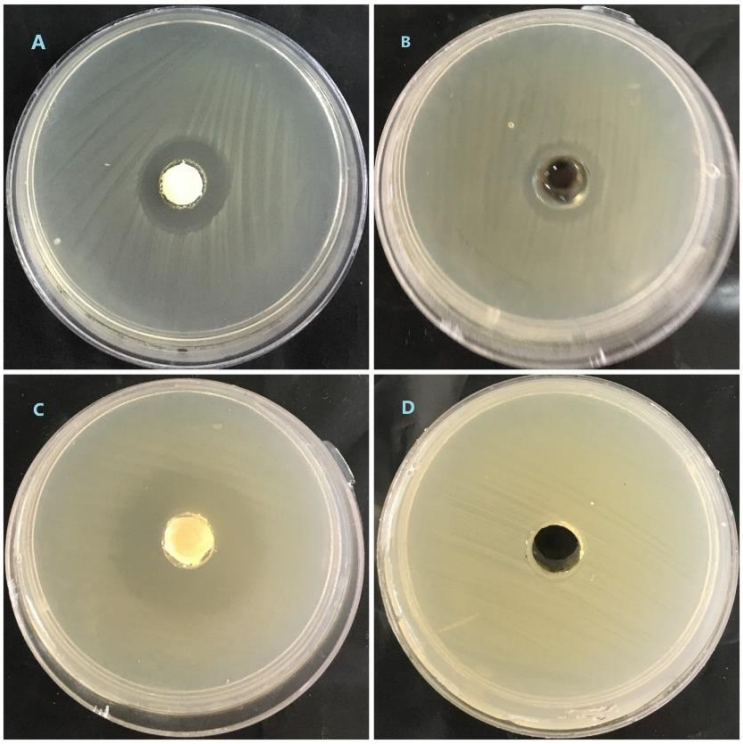

Figure 1. The non-growth halo of Enterococcus faecalis bacteria in the vicinity of the tested materials.
A. AH Plus sealer, B. Lactobacillus casei supernatant, C. Combination of Lactobacillus casei supernatant and AH plus sealer, D. Negative control

Table 1. The mean diameters of the non-growth halo in millimeters at different times

Group	After 24 h Mean±SD	After 48 h Mean±SD	After 72 h Mean±SD	p-value
AH Plus sealer	17.16±1.04[a]	15.75±1.08[a]	14.5±1.32[a]	0.079
Lactobacillus casei supernatant	14.66±0.57[a]	14.16±1.04[a]	13.66±1.52[a]	0.578
Combination of lactobacillus casei supernatant and AH Plus sealer	27.33±1.25[a]	24.66±1.15[a]	24.33±0.76[a]	0.051

a: Similar Latin letters indicate that there is no significant difference between groups.

The rate of biofilm formation in Enterococcus faecalis after treatment with Lactobacillus casei supernatant, AH Plus sealer and their combination has decreased compared to the positive control well (Figure 2). The inhibition percentage of Enterococcus faecalis biofilm formation after treatment with AH

Plus sealer, Lactobacillus casei supernatant, and the combination of these two were 23%, 17% and 33%, respectively. The reduction of biofilm formation in the groups treated with AH Plus sealer and the combination of supernatant with AH Plus sealer, compared to the control group, decreased significantly ($p<0.05$). However, there was no significant difference in the reduction of biofilm formation between the control group and Lactobacillus casei supernatant.

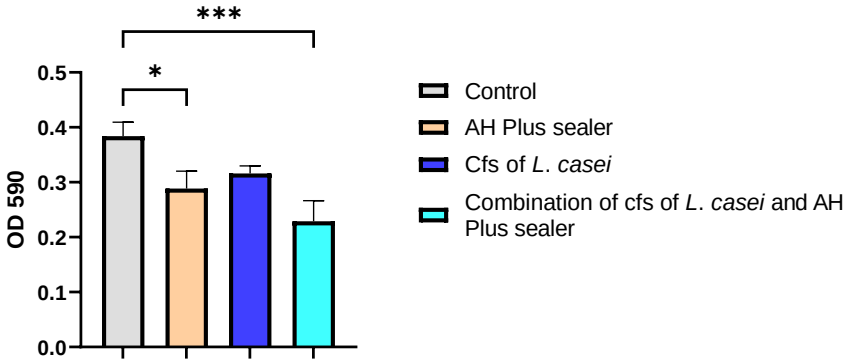

Figure 2. The effect of Lactobacillus casei supernatant, AH Plus sealer and the combination of Lactobacillus casei supernatant and AH Plus sealer on Enterococcus faecalis biofilm formation ($p<0.001^{***}$, $p<0.05^{*}$)

Discussion

The results of this study showed that the combination of Lactobacillus casei supernatant and AH Plus sealer has more antibacterial and anti-biofilm effects than either of them alone on Enterococcus faecalis. The use of this compound led to an increase in the inhibition of bacterial growth and maintaining the diameter of the no-growth halo even after 72 hours of incubation. Furthermore, the results showed that both substances independently have anti-biofilm properties, but their combination increased the inhibition of Enterococcus faecalis biofilm formation.

In the present study, the mean diameter of the non-growth halo of the standard strain of Enterococcus faecalis in the presence of Lactobacillus casei supernatant after 24, 48 and 72 hours of incubation was 14.66, 14.16 and 13.66 mm, respectively. The study of Tajehmiri et al. showed that the non-growth halo diameter of Shigella dysentery PTCC 1188, Salmonella enterica PTCC 1231, Escherichia coli PTCC 1399 and Staphylococcus aureus PTCC 1431 in the presence of Lactobacillus casei supernatant after 48 hours of incubation were 11.5, 11, 11.5 and 8.5 mm, respectively (21). Moreover, Rocha-Ramírez et al. showed a non-growth halo diameter of 10 to 20 mm against EAEC and EHEC strains and Escherichia coli strain ATCC 25922 in the presence of Lactobacillus casei supernatant after 24 hours of incubation (22). The difference in the non-growth halo diameter of bacteria in the presence of the supernatant prepared in this study compared to other studies is due to the difference in the type of studied bacteria and the type of Lactobacillus casei strain.

In the present study, the mean non-growth halo diameter of Enterococcus faecalis in the presence of AH Plus sealer after 24, 48 and 72 hours of incubation was 17.16, 15.75 and 14.5 mm, respectively. According to the results of a study by Donyavi et al., the mean non-growth halo diameter of Enterococcus faecalis bacteria in the vicinity of AH Plus sealer after 24, 48, and 72 hours of incubation was 17.3, 14, and 8.03 mm, respectively (23). The results of a study by Rathod et al. showed that the mean non-growth halo diameter of Staphylococcus aureus and Candida albicans in the presence of AH Plus sealer after 24

incubations was 18.99 and 21.97 mm, respectively (16). This is attributed to the release of formaldehyde from the sealer until it is fully cured. In the present study, data analysis based on different times showed that the increase in time led to a decrease in the diameter of the AH Plus sealer containment area. But due to the combination of sealer with the supernatant of Lactobacillus casei, the non-growth halo diameter after 72 hours of incubation was reduced by a smaller amount. It can be concluded that due to the short-term release of formaldehyde from the sealer, the reduction of the antimicrobial effect of AH Plus sealer is due to the effect of Lactobacillus casei supernatant.

The results of the present study showed that the supernatant of Lactobacillus casei prevents the formation of biofilm by Enterococcus faecalis with an average of 23%. In a study by Jaffar et al., after 24 hours of incubation, approximately 90% biofilm inhibition activity was observed for Lactobacillus casei supernatant against Aggregatibacter actinomycetemcomitans strain (24). In another study, supernatants of faecal lactobacilli caused an 85-95% reduction in biofilm formation of Vibrio cholerae isolates (25). These differences may be due to differences in the type of bacteria investigated, laboratory conditions and the strength of probiotics used.

The results of the present study showed that AH Plus sealer prevents biofilm formation by an average of 17%. In this context, Khot evaluated the ability of AH Plus sealer to inhibit biofilm, and the results showed that sealer prevents the formation of biofilm by Enterococcus faecalis (26). Becker et al. evaluated the anti-biofilm activity of epoxy sealer with tetravalent ammonium against Enterococcus faecalis, and the results showed a 25% and 75% reduction in biofilm formation at different concentrations of ammonium (27). Zordan-Bronzel et al. reported the inhibition percentage of Enterococcus faecalis biofilm after 24 hours of contact with AH Plus sealer extract as 15.9% (20). These differences may be attributed to various factors including laboratory conditions, type of sealer and concentrations used in these studies.

The results of this study showed that AH Plus sealer alone has more antibacterial properties than Lactobacillus casei supernatant, while Lactobacillus casei supernatant had better anti-biofilm properties. However, the combination of these two substances led to an increase in both antibacterial and anti-biofilm properties. These results show that the use of Lactobacillus casei supernatant as a complementary combination with sealers can help improve their antimicrobial effects. It is suggested that in future studies, the antibacterial effect of other sealers alone and in combination with probiotics on Enterococcus faecalis and other oral pathogens be investigated.

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