



## Good Bugs vs Bad Bugs: Evaluation of Inhibitory Effect of Selected Probiotics against *Enterococcus faecalis*

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### ABSTRACT

**Introduction:** The main goal of endodontics is the prevention of apical periodontitis. This is due to the presence of persistent pathogenic microorganisms, such as *Enterococcus faecalis*, and its ability to directly cause acute and chronic inflammation in the periapical tissues. *Lactobacillus* has been shown to promote health in the intestines as well as to inhibit the growth of certain problematic oral bacteria. This study explores shifting the established paradigm of endodontic treatment, which has focused on eliminating all bacteria from the canal system and on elimination of the problematic bacteria through introducing probiotics. A preliminary work was performed to evaluate the possible effectiveness of probiotics in preventing the growth of *E. faecalis*.

**Materials and methods:** Two probiotic cultures *Lactobacillus plantarum* ATCC 8041 and *Lactobacillus rhamnosus* ATCC 7408 were selected to check their antimicrobial activity against *E. faecalis* ATCC 29212 by two methods: agar cup/well diffusion method and deferred antagonism test.

**Agar cup method:** A total of 0.5 mL of requisite test pathogen culture was inoculated into 20 mL of molten sterile Mueller and Hinton agar and cooled to  $45 \pm 2^\circ\text{C}$ . Circular wells of diameter 10 mm were punched in each of the poured plates. Appropriately diluted test samples were added to the above-punched wells. The plates were incubated upright position at  $37^\circ\text{C}$  for 24 hours in aerobic conditions. Postincubation, zone of inhibition was measured. The cell-free supernatant of *Lactobacillus* species was also evaluated for antimicrobial activity.

**Deferred antagonism test:** The test probiotic strain was standardized to 0.1 optical density (OD) at 600 nm and inoculated in a 1 cm wide diametric streak across the surface of trypticase

soy agar + yeast extract + calcium carbonate (TSYCa) agar using a sterile cotton swab. Then, the plate agar containing the test strain was incubated at  $37^\circ\text{C}$  for 24 hours under aerobic condition and then standardized to 0.1 OD at 600 nm for overnight (18 hours,  $37^\circ\text{C}$ ). A purified culture of indicator strain (pathogen) was streaked at right angles to the line of original producer growth. Postincubation plates were observed for the inhibition zone width of the indicator strain.

**Results:** Under the conditions of this study, *Lactobacilli* had an inhibitory effect on the growth of *E. faecalis* by agar cup method but not by deferred antagonism test.

**Conclusion:** This pilot study demonstrated that probiotics show a potential in root canal therapy.

**Clinical Significance:** If probiotics are effective against endodontic pathogens, they can be potentially used as intracanal medicaments. This will be a novel concept of introducing bacteriotherapy in endodontics and replacing pathogenic bacteria by healthy bacteria, normal flora.

**Keywords:** Antimicrobial, Bacteria, Bacteriotherapy, Endodontics, Probiotics.

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### INTRODUCTION

Simply defined, probiotics are live bacteria that confer a health benefit to the host. The term "probiotic" was initially coined to oppose the term "antibiotic" by Lilley and Stillwell in 1965. The World Health Organization recognizes probiotics as to be the next most important immune defense system in the event that current antibiotics become useless due to the development of bacterial resistance. This concept has fuelled research for using probiotics in medicine and dentistry.

The organisms that have been used in the past as probiotics have been certain strains of *Lactobacillus* and

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*Bifidobacteria*.<sup>1</sup> Some of the mechanisms of action of these probiotics include the productions of bacteriocin-like inhibitory substances (BLIS) and the altering of the local pH, competing for nutrients, forming physical barriers, and stimulating the immune response.<sup>2</sup> Within the field of endodontics, the traditional method of treatment is to eliminate the bacteria present in the root canal system. When root canal therapy fails, the presence of bacteria is almost always present. *Enterococcus faecalis* is a particular strain of bacteria, i.e., found consistently in posttreatment endodontic disease.<sup>3</sup> Endodontic therapy aims to remove bacteria through chemomechanical methods.

As far as is known, probiotics work is sparse in terms of their therapeutic effect in the treatment of endodontic disease. This study was carried out to check if certain probiotics would have an inhibitory effect against a known endodontic pathogen. We proposed to grow known probiotics in the presence of the established postendodontic treatment disease bacteria *E. faecalis* and observe what effect the probiotics would have against it. We hypothesized that the probiotics *L. plantarum* and *L. rhamnosus* would have an inhibitory effect against *E. faecalis*.

## MATERIALS AND METHODS

### Study Design

To test our hypothesis, we designed an *in vitro* study, which would attempt to grow certain probiotic microorganisms along with the known endodontic pathogen (*E. faecalis*) and observe if the probiotic had an inhibitory effect on the endodontic pathogen. The inhibitory activity was tested with two methods: Agar cup method and deferred antagonism test.

### Test Groups

The probiotics studied were of two strains: *L. plantarum* ATCC 8041 and *L. rhamnosus* ATCC 7408. It is a Gram-positive facultative anaerobic rod-shaped bacteria. The endodontic pathogen used in this study was *E. faecalis* ATCC 29212. It is a Gram-positive facultative anaerobic cocci.

### Agar Cup Method

About 0.5 mL of requisite test pathogen culture of 0.1 optical density (OD) at 620 nm was inoculated into 20 mL of molten sterile agar (Mueller and Hinton agar) and cooled to  $45 \pm 2^\circ\text{C}$ . Afterward, it is mixed thoroughly and poured into a sterile empty Petri dish and allowed to solidify. Circular wells of diameter either 10 mm were punched with a sterile steel cork borer in each of the poured plates. Appropriately, diluted test samples were added to the above punched wells. The volume of

the solution added to each well was 150  $\mu\text{L}$ . The plates were incubated in upright position at  $37^\circ\text{C}$  for 24 hours under aerobic conditions. Postincubation, zone of inhibition was measured. The inhibitory activity of test strain is considered significantly positive if the zone of inhibition produced by the test strain (probiotic strain) against the indicator strain (endodontic pathogen) is at least 10 mm.

### Extraction of Cell-free Supernatant

The pure isolate of *Lactobacillus spp.* was propagated in 100 mL flask containing MRS broth (pH 6.0) and then incubated at  $37^\circ\text{C}$  for 72 hours under microaerophilic conditions. The supernatant that may contain crude bacteriocin, a cell-free solution, was obtained by centrifuging the culture at 10,000 rpm for 20 minutes at  $4^\circ\text{C}$ . After centrifugation, the supernatant was collected in a fresh sterile tube and pellet was discarded. The cell-free supernatant (CFS) was passed through 0.22  $\mu\text{m}$  membrane filter and was evaluated for any antimicrobial activity. The crude CFS adjusted to pH 6.0 using 1N NaOH prior testing and crude CFS was used as well. The crude CFS diluted to 1:2 was used for testing.

### Deferred Antagonism Test

The test probiotic strain was standardized to 0.1 OD at 600 nm and inoculated using a sterile cotton swab in a 1-cm wide diametric streak across the surface of TSYCa agar (trypticase soy agar + yeast extract + calcium carbonate). The plate agar containing the test strain was then incubated at  $37^\circ\text{C}$  for 24 hours under microaerophilic condition. Macroscopically, visible growth was removed by scraping with the edge of a glass slide. This plate was inverted over a circle of chloroform-soaked filter paper in the lid of a Petri dish. After 30 minutes, the plate was removed from the lid and was exposed to the air for 15 minutes. Then, standardized to 0.1 OD at 600 nm of overnight (18 hours,  $37^\circ\text{C}$ ) and purified culture of indicator strain (pathogen) was streaked at right angle to the line of original producer growth with a cotton swab. The same procedure was done in triplicates and all the TSYCa agar plates with the test and indicator strains were incubated at  $37^\circ\text{C}$  for 24 hours aerobically. Postincubation, plates were observed for the inhibition zone width of the indicator strain.

## RESULTS

### Results of AgarCup/Well Diffusion Method

The CFS of *L. plantarum* and *L. rhamnosus* were tested against *E. faecalis* ATCC 29212 using agar cup/well diffusion method (Fig. 1 and Table 1). The CFS of the two strains was used in three forms: (a) Neat/crude; (b) CFU diluted to 1:2; (c) CFS adjusted to pH 6.0.

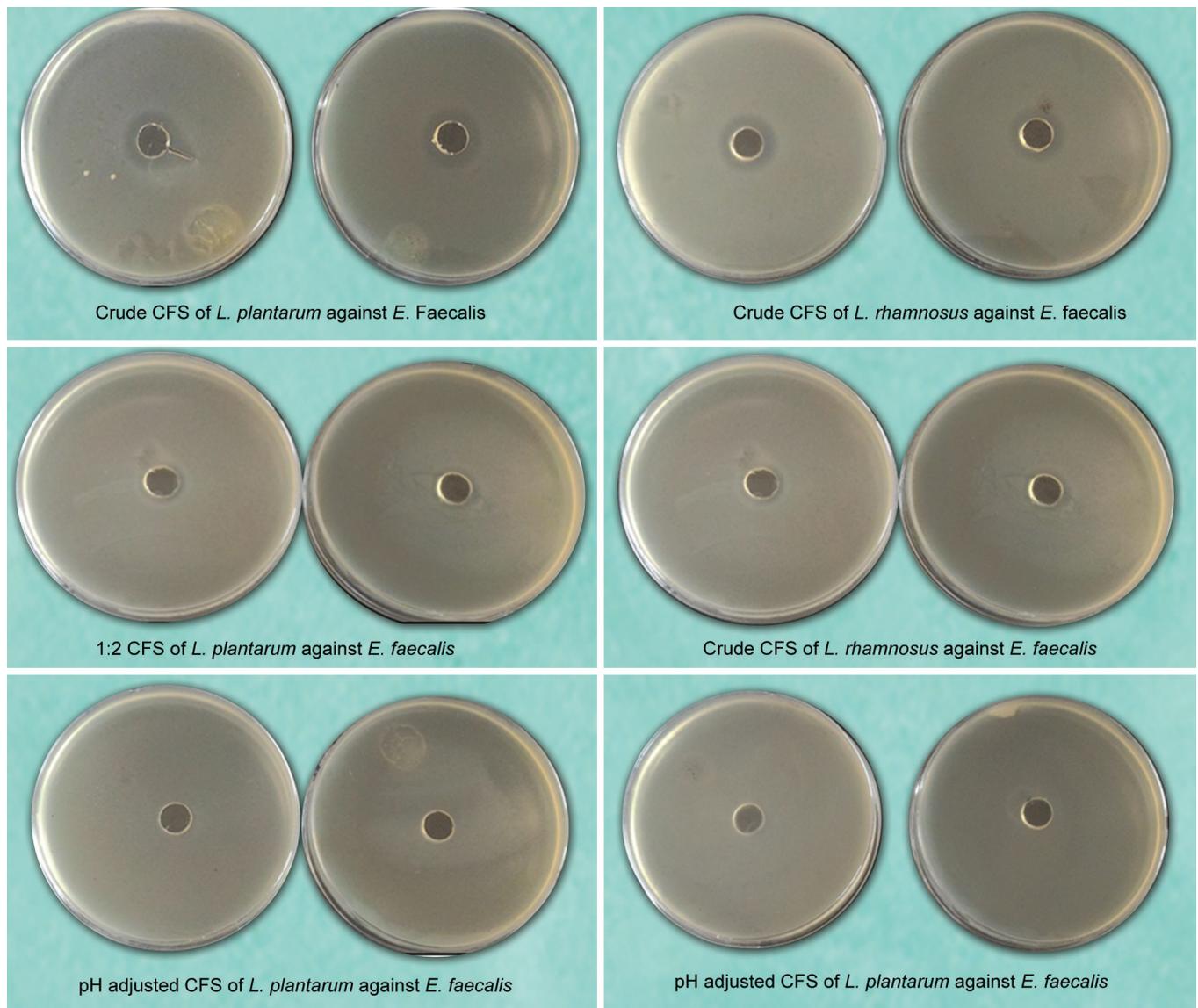


Fig. 1: Agar cup method test of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* against *Enterococcus faecalis*

Table 1: Zone of inhibition of test strain (*Lactobacilli*) against indicator strain (*E. faecalis*) by agar cup method

Sample concentration	Zone of inhibition in mm <i>E. faecalis</i> ATCC 29212	
	n = 1	n = 2
Crude CFS of <i>L. plantarum</i>	20	20
Crude CFS of <i>L. rhamnosus</i>	20	19
1:2 CFS of <i>L. plantarum</i>	No zone	No zone
1:2 CFS of <i>L. rhamnosus</i>	No zone	No zone
Crude CFS of <i>L. plantarum</i> pH adjusted to 6.0	No zone	No zone
Crude CFS of <i>L. rhamnosus</i> pH adjusted to 6.0	No zone	No zone

- The crude CFS of both *L. plantarum* and *L. rhamnosus*, when tested against *E. faecalis* ATCC 29212, showed antimicrobial activity with an average zone of inhibition of 20 mm.
- The 1:2 diluted, crude CFS of both strains when tested against *E. faecalis* ATCC 29212 demonstrated no antimicrobial activity.

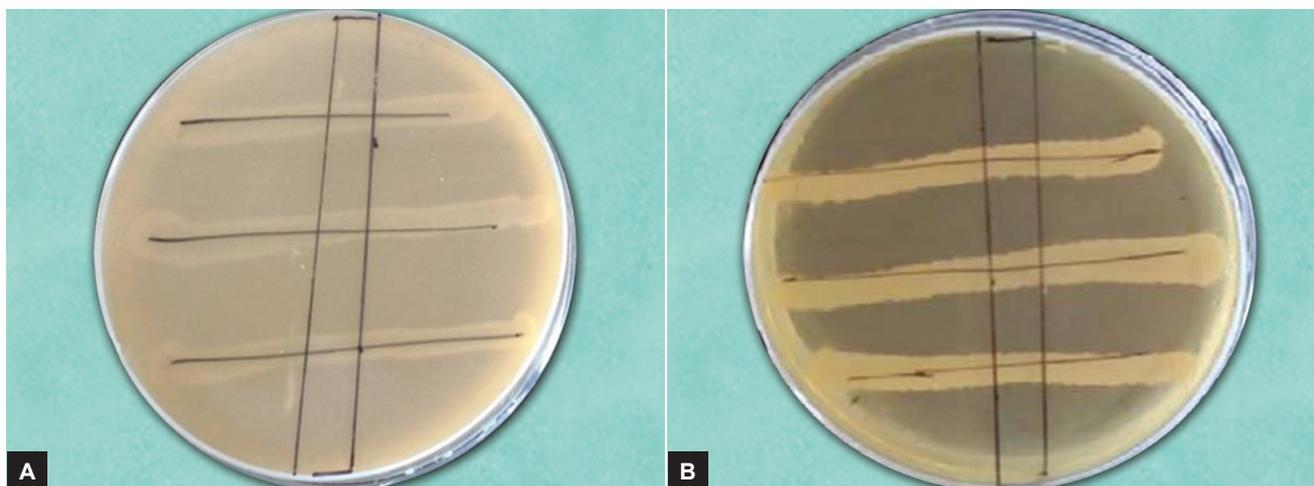
- Furthermore, when the crude CFS was adjusted to pH 6.0, showed no activity against *E. faecalis* ATCC 29212.

### Results of Deferred Antagonism Test

*Lactobacillus plantarum* and *L. rhamnosus* showed variable results; no zone of inhibition was observed against indicator strain, and therefore, it could not be concluded that they are fully effective against the specific pathogenic organism tested (Table 2, Figs 2A and B).

Table 2: Zone of inhibition of test strain (*Lactobacilli*) against indicator strain (*E. faecalis*) by deferred antagonism method

Test strain	Zone diameter (mm) <i>E. faecalis</i> ATCC 29212		
	n = 1	n = 2	n = 3
<i>L. plantarum</i>	No zone	No zone	No zone
<i>L. rhamnosus</i>	No zone	No zone	No zone



**Figs 2A and B:** Activity of (A) *Lactobacillus plantarum* against *Enterococcus faecalis*; and (B) *Lactobacillus rhamnosus* against *E. faecalis* in deferred antagonism test

## DISCUSSION

Probiotic use has been studied for the treatment of oral health problems. Specifically, the use of probiotics has been explored to aid in the treatment of periodontal problems, halitosis, and caries prevention.<sup>2,4</sup> The literature suggests that most commonly used strains are *Lactobacillus* and *Bifidobacterium* genera, commonly found in the oral cavity, including caries lesions.<sup>5</sup> These were the first probiotic species to be introduced into research.<sup>6</sup> *Lactobacillus rhamnosus* GG, ATCC 53103 has been proposed to reduce the risk for caries by producing a growth inhibitory substance against *Streptococcus sobrinus*.<sup>5</sup> Other strains of probiotics in the oral cavity include: *L. acidophilus*, *L. casei* Shirota, *L. paracasei*, *L. casei*, *L. johnsonii*, *L. reuteri*, *Propionibacterium*, and *Weisellacibaria*.<sup>7</sup>

The probiotic approach has not yet been extensively evaluated for use in endodontic therapy. Endodontics is the branch of dentistry, i.e., concerned with the morphology, physiology, and pathology of the human dental pulp and periradicular tissues. It has been established that the primary etiology of endodontic infections is bacteria.<sup>8-10</sup> Primary infections of the necrotic pulp tissue are generally composed of a mixed bacterial community dominated by anaerobic Gram-negative bacteria.<sup>11</sup> Persistent infections tend to be dominated by a more specific community of bacteria. These bacteria are anaerobic and Gram positive, in particular *E. faecalis*.<sup>12</sup>

*Enterococcus faecalis* appears to be highly resistant to the medicaments used in the treatment, and it is one of the few microorganisms shown *in vitro* to be resistant to calcium hydroxide due to its proton pump.<sup>13</sup> *Enterococcus faecalis* establishes an endodontic infection and maintains a periradicular inflammation due to its virulence factors.<sup>14</sup> It is also able to survive as a single organism without the support of other bacteria. In addition, *E. faecalis* has the ability to form a surface-attached microbial community

known as a biofilm. This allows it to be protected from host defenses as well as systemic treatment. Different methods of combating *E. faecalis* were explored through the use of different irrigants. "Out of the box" treatments were evaluated for use against *E. faecalis*, such as the use of passion fruit juice as an endodontic irrigant<sup>15</sup> as well as the use of lasers and phytomedicine.<sup>16,17</sup> Probiotics hold a potential avenue of more common and broad use for antibacterial treatment in the future. In this study, an innovative approach that might aid in increasing success of endodontic therapy was investigated. This innovative approach involves bacteriotherapy by allowing probiotic organisms to eliminate pathogenic organisms, either by outcompeting/immune modulation or by secreting antimicrobial substances, such as peroxides. In this pilot study, the antimicrobial activity of probiotics is determined by two methods: Agar cup/well diffusion method and deferred antagonism against the endodontic pathogen. Under the conditions of this study, *Lactobacilli* had an inhibitory effect on the growth of *E. faecalis* by agar cup method but not by deferred antagonism test. In well-diffusion method, the inhibition zone was about 20 mm against *E. faecalis* and this showed that the agar cup method was more appropriate as compared to the other method. As a result, the inhibitory effect produced by test strain against *E. faecalis* was well observed. These results are further supported by study done by Seifelnasr.<sup>18</sup>

The method of "deferred antagonism" has been used to evaluate the antibacterial properties of the normal flora of the nasopharynx.<sup>19</sup> Bacteriocin-like inhibitory substances are used to describe the bacterial products that have inhibitory effects. The deferred antagonism test is used to look for BLIS production. This test works by testing two bacteria to see their effects on one another without direct contact between them. The BLIS proteins secreted by the beneficial bacteria would diffuse through

the agar and produce inhibition away from the location of the bacteria that secreted them.

## CONCLUSION

This pilot study demonstrated that probiotics show a potential in root canal therapy. Despite great promises, endodontic works are sparse and need validation by further *in vitro*, *in vivo* research and large randomized trials. This will determine the full potential of “bacteriotherapy” and its application in endodontics.

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