The Starvation Resistance and Biofilm Formation of *Enterococcus faecalis* in Coexistence with *Candida albicans*, *Streptococcus gordonii*, *Actinomyces viscosus*, or *Lactobacillus acidophilus*

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**Abstract**

**Introduction:** *Enterococcus faecalis* is the most frequently detected species in root canal–treated teeth, and it is able to survive under starvation conditions. However, persistent periapical disease is often caused by multispecies. The aim of this study was to explore the survival of *E. faecalis* in starvation conditions and biofilm formation with the 4 common pathogenic species. **Methods:** A dual-species model of *Candida albicans*, *Streptococcus gordonii*, *Actinomyces viscosus*, or *Lactobacillus acidophilus* in combination with *E. faecalis* was established and allowed to grow in phosphate-buffered saline for the examination of starvation survival. Cefuroxime sodium and vancomycin at a concentration of 100 mg/L were added into brain-heart infusion plate agar to count the 2 bacteria separately in the dual species. Scanning electron microscopy was used to observe the dual species and multiple species on the root canal dentin of bovine teeth for 48 hours. A confocal laser scanning microscope was used to show the 4 groups of dual-species biofilms on substrates with glass bottoms for 48 hours. **Results:** *E. faecalis* was more resistant to starvation in coexistence with *C. albicans*, *S. gordonii*, *A. viscosus*, or *L. acidophilus*, and *S. gordonii* was completely inhibited in coexistence with *E. faecalis*. The dual-species biofilm showed that *E. faecalis* formed thicker and denser biofilms on the root canal dentin and glass slides in coexistence with *S. gordonii* and *A. viscosus* than *C. albicans* and *L. acidophilus*. **Conclusions:** The multispecies community is conducive to the resistance to starvation of *E. faecalis* and biofilm formation in root canals. (J Endod 2016;42:1233–1238)

**Key Words**

Biofilm, dual species, *Enterococcus faecalis*, starvation

**Significance**

Pathogenic bacteria are a major cause of endodontic infections and periapical periodontitis. Microorganisms in the root canal are involved in dynamic processes, and bacterial species depend on available nutrition, oxygen levels, and local pH in the root canal system. In a primary root canal infection, the open root canal provides an available resource for microbes through direct communication with the oral cavity. Anaerobic gram-negative bacteria and several gram-positive rods are often found in the root canal. However, in root-filled canals with persistent periapical disease, an obvious decrease in the availability of resources in the root canal occurs because of the preparation and intracanal medication are introduced and the root canals are sealed. Gram-negative bacteria gradually decrease, and gram-positive bacteria are more frequently present, including streptococci, *Parvimonas micra*, *Actinomyces* species, lactobacilli, *Propionibacterium* species, and *Enterococcus* (3–7). A few studies have indicated that *Enterococcus faecalis* is the most frequently detected species in root canal–treated teeth, and it promotes persistent disease (8–11). *E. faecalis* has the ability to adapt to harsh environmental changes, such as an extreme alkaline pH, salt concentrations, deprivation of nutrition, antimicrobial resistance, and growth in the root canal as a biofilm (12, 13). The properties of *E. faecalis* determine its survival in root-filled canals. In the past, the frequent occurrence of monocultures of *E. faecalis* has raised suspicion that this bacterium may be the sole organism that persists in root canals, but monoinfections seldom occur in nature. Chronic periapical disease is often caused by multiple species, and the interactions of these species are very complex (14, 15). Interspecies interactions in multispecies bacterial biofilms exhibit social behavior and generate the protective effect of...
biofilms (16). *E. faecalis* frequently inhabits this undernourished environment of root-filled canals and depends not only on its own properties but also the synergistic and antagonistic interactions between species in the biofilm. At present, the interspecies interactions of multispecies biofilms in root-filled canals are still poorly understood, and further information on interspecies interactions is needed.

The root-filled canal is an environment with deficient nutrition, and microbes are grown in biofilms in root canals. *Lactobacillus*. *Actinomyces*, *Streptococcus*, and *Candida* have been reported to be pathogenic species that are isolated in retreated root canals (2, 17, 18). In this study, dual-species models of *E. faecalis* and *Lactobacillus acidophilus*, *Actinomyces viscosus*, *Streptococcus gordoni*, or *Candida albicans* were used to evaluate their resistance to starvation, and the biofilm structure was observed with scanning electron microscopy and a confocal laser scanning microscope.

**Materials and Methods**

**Bacterial Culture**

*E. faecalis* ATCC 29212, *C. albicans* ATCC 90028, *L. acidophilus* ATCC 4356, *A. viscosus* ATCC 15987, and *S. gordoni* ATCC 10558 were used in this study. The bacteria were streaked from a frozen stock culture onto their respective plates at 37°C for 48 hours. *E. faecalis*, *A. viscosus*, and *S. gordoni* were grown on brain-heart infusion agar (BHI; Difco Laboratories, Detroit, MI) plates; *C. albicans* were grown on yeast peptone dextrose agar (Difco) plates; and *L. acidophilus* were grown on de Man, Rogosa, and Sharp agar (Difco) plates. A single colony of bacteria was inoculated into 5 mL of the corresponding liquid broth and cultured at 37°C under anaerobic conditions up to the exponential phase.

**Specimen Preparation**

Bovine incisors were used to measure the biofilm formation of dual species on root canal dentin. Fresh bovine incisors were extracted from animals that were slaughtered for commercial purposes in a slaughterhouse in China and stored in saline. Our study exerted no influence on the fate of the animals at any point. The specimen preparations were similar to those described in a previous study (19). Briefly, the crown and root apex of the teeth were removed, and the length of the remaining tooth root was 10 mm. Access preparations were instrumented to a size 60 accompanied by irrigation with 5.25% sodium hypochlorite and 17% EDTA to remove the pulp tissue and the smear layer. The roots were longitudinally split with 5.25% sodium hypochlorite and 17% EDTA to remove the pulp tissue and the smear layer. The roots were longitudinally split into 2 parts, and the root canal dentin was exposed to bacteria to allow biofilm formation. The prepared specimens were sterilized in an autoclave at 121°C for 20 minutes before use as a substrate in the dual-species coculture.

**Antibacterial Assay**

According to the different bacterial resistance to antibiotics, 100 mg/L cefuroxime sodium was added to BHI agar to distinguish *E. faecalis* and *A. viscosus*, *E. faecalis* and *L. acidophilus*, or *E. faecalis* and *S. gordoni*, and 100 mg/L vancomycin was added to BHI agar to distinguish *E. faecalis* and *C. albicans* in dual-species coculture. The minimum inhibitory concentration (MIC) was determined as described in a previous study (20). In short, 10 μL bacterial culture, 140 μL BHI broth, and 50 μL 2-fold diluted antibiotics were added to 96-well microplates and cultured at 37°C under anaerobic conditions for 24 hours. The MIC is defined as the lowest concentration of an antibiotic that inhibits bacterial growth with an increase in optical density <0.050 under standard conditions. The MIC was determined at least in triplicate on a different day.

**The Starvation Assay of Dual Species**

In this assay, *E. faecalis* and *C. albicans* (EC), *E. faecalis* and *S. gordoni* (ES), *E. faecalis* and *A. viscosus* (EA), and *E. faecalis* and *L. acidophilus* (EL) established 4 dual-species models. In a mixture of dual species, the final concentrations of both bacteria were adjusted to 10^8 colony-forming units (CFUs)/mL according to their respective growth curves. The bacterial culture of the dual species was centrifuged at 5000 rpm at 4°C for 5 minutes. The supernatant was discarded. The cell deposit was washed twice with PBS, and then resuspended in PBS for the starvation assay of dual species. The total bacterial amount of the dual species was obtained on 1, 2, 4, 7, 14, and 28 days of starvation by bacterial plate counts. At each time point of starvation, 100 μL dual species was 10-fold diluted and spread on BHI agar for 48 hours of incubation. The total bacterial amount of dual species was counted. Meanwhile, to separately count *E. faecalis* in the ES, EA, and EL groups, 100 μL dual species was 10-fold diluted and spread on BHI agar containing 100 mg/L cefuroxime sodium only to allow *E. faecalis* colony formation. To count *C. albicans* separately in the EC group, 100 μL dual species was 10-fold diluted and spread on BHI agar containing 100 mg/L vancomycin only to allow *C. albicans* colony formation. The bacterial count of the other species in the dual-species model was calculated by subtracting the bacterial quantity in BHI agar with antibiotics from the total bacterial quantity. All determinations were performed at least 3 times on different days.

**Root Canal Infection with Dual Species**

Root canal infections with dual-species biofilm were performed under the condition of abundant nutrition because starvation affects bacterial growth and does not form a typical biofilm structure. The EC, ES, EA, and EL groups and a group of mixture of the 5 bacteria (*E. faecalis*, *C. albicans*, *S. gordoni*, *A. viscosus*, and *L. acidophilus*) were inoculated into 12-well plates (Corning Costar, Corning, NY) containing 3 mL BHI broth, respectively, and the final concentration of each bacteria was 10^5 CFU/mL. Five root specimens were transferred to each well with inoculated culture medium and incubated at 37°C for 48 hours. The specimens were rinsed twice with sterile PBS to remove planktonic and loosely adherent bacteria and to ensure that only the attached biofilm remained. The specimens were prepared for scanning electron microscopy (SEM) (E-1010; Hitachi, Ibaraki, Japan) by fixation in 2.5% glutaraldehyde and dehydration in a series of acetone solutions (50%, 70%, 80%, and 90% for 20 minutes each and 100% for 20 minutes twice). The specimens were dried and coated with gold, and a randomly selected area of biofilm was observed on the root canal dentin using SEM.

**Confocal Laser Scanning Microscopy**

The bacterial competition in dual-species biofilms was observed under the condition of abundant nutrition using a confocal laser scanning microscope (CLSM). Petri dishes with glass bottoms (diameter = 35 mm [Hangzhou Shengyou Biotechnology, Zhejiang, China]) were considered to be a substrate to allow biofilm formation. The EC, ES, EA, and EL groups of dual species at a 1:1 bacterial ratio were added into the Petri dishes with 2 mL tryptic soy broth (Difco) supplemented with 1% glucose, and the final concentration of each bacteria was 10^6 CFU/mL. After 48 hours of incubation at 37°C, the biofilm at the bottom of the well was gently washed twice with sterile PBS and stained with a mixture of 6 μmol/L SYTO 9 stain and 30 μmol/L propidium iodide (PI) at room temperature in the dark for 15 minutes according to the specifications of the L13152 kit (LIVE/DEAD Bac-Light Bacterial Viability Kit; Molecular Probes, Eugene, OR). Images of the stained specimens were captured using a Carl Zeiss CLSM (Carl Zeiss,
MicroImaging Inc, Thornwood, NY) and ZEN software (ZEN 2012 Light Edition, Carl Zeiss MicroImaging, Inc). SYTO 9 and PI were excited at 488 nm and 543 nm, respectively. The 3-dimensional (3D) bacterial biofilm was scanned along the z-axis. All images are shown at 400× magnification. SYTO 9 stain generally labels all bacteria in a population as those with intact membranes and those with damaged membranes. In contrast, PI penetrates only bacteria with damaged membranes, causing a reduction in SYTO 9 stain fluorescence when both dyes are present. Therefore, with a mixture of the SYTO 9 and PI stains, viable bacteria with intact cell membranes stain fluorescent green, whereas dead bacteria with damaged membranes stain fluorescent red.

Statistical Analysis

Statistical analysis was performed using SPSS 18.0 software (SPSS Inc, Chicago, IL). Raw bacterial colony counts were transformed to log10 values to normalize the data. One-way analysis of variance and the Tukey honestly significant difference test were used to compare the survival rates of E. faecalis in the 4 groups of dual species and alone at the time of starvation and the biofilm thickness of the 4 groups of dual species and E. faecalis alone for 48 hours. The Student t test was used to compare the survival rates of C. albicans, S. gordonii, A. viscosus, and L. acidophilus in dual species and alone at the same time of starvation. P < .05 was considered statistically significant.

Antibacterial Assay

Vancomycin and cefuroxime sodium showed different antibacterial activities against E. faecalis, C. albicans, L. acidophilus, A. viscosus, and S. gordonii (Table 1).

Dual-species Resistance to Starvation

At 1 and 2 days of starvation, no significant difference was found between the survival rates of E. faecalis in dual species and alone (P > .05). E. faecalis generated greater resistance to starvation in the presence of C. albicans, S. gordonii, A. viscosus, and L. acidophilus than alone after 4 days, and both showed significant differences (P < .05). The coexistence of C. albicans significantly improved the survival rates of E. faecalis in starvation conditions (Fig. 1).

In the starvation evaluation of dual species, C. albicans had a good ability to endure starvation whether in coexistence with E. faecalis or alone, and both showed no statistical difference at the same day of starvation (P > .05) (Fig. 2A). E. faecalis completely inhibited S. gordonii under starvation conditions in dual species, and S. gordonii did not survive for 1 day of starvation in the presence of E. faecalis. The survival rates of S. gordonii showed a statistically significant difference in the presence of E. faecalis or alone (P < .05) (Fig. 2B). In the dual species of E. faecalis and A. viscosus, the survival rates of A. viscosus showed a relative rise in comparison with them alone (Fig. 2C). Enduring starvation, the survival rate of L. acidophilus did not show a significant change in the presence of E. faecalis, but at 28 days of starvation, L. acidophilus in coexistence with E. faecalis showed a lower survival rate than alone (Fig. 2D).

Biofilm Morphology of Dual Species in Root Canals

The biofilm structure of the 4 groups of dual species and a group of multiple species in the root canal were examined by SEM. The coculture of E. faecalis and C. albicans (Fig. 3A), E. faecalis and L. acidophilus (Fig. 3D), and a mixture of 5 strains (Fig. 3E) showed a relatively thin biofilm on the root canal dentin, and the dentinal tubules were still clearly recognized. Both E. faecalis and C. albicans were observed on the root canal dentin in coculture (Fig. 3A), whereas in the coculture of E. faecalis and L. acidophilus, E. faecalis dominated the root canal dentin, and only a small quantity of L. acidophilus was observed (Fig. 3D). In the biofilm structure of the coculture of 5 strains, cocci, bacilli, and Candida appeared on the root canal dentin (Fig. 3E). E. faecalis and S. gordonii (Fig. 3B) and E. faecalis and A. viscosus

Table 1. Minimum Inhibitory Concentration of 5 Test Bacteria against Vancomycin and Cefuroxime Sodium

<table>
<thead>
<tr>
<th>Antibiotics (mg/L)</th>
<th>E. faecalis</th>
<th>C. albicans</th>
<th>A. viscosus</th>
<th>L. acidophilus</th>
<th>S. gordonii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>8</td>
<td>&gt;1024</td>
<td>2</td>
<td>&gt;1024</td>
<td>1</td>
</tr>
<tr>
<td>Cefuroxime sodium</td>
<td>512</td>
<td>&gt;1024</td>
<td>&lt;0.5</td>
<td>4</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

Figure 1. The survival count of E. faecalis alone and in coexistence with C. albicans, S. gordonii, A. viscosus, and L. acidophilus, respectively, for 1 to 28 days of starvation. The asterisks represent a statistically significant difference between the survival count of E. faecalis in dual species and alone at the same day of starvation (P < .05).
(Fig. 3C) formed a rather thick and dense biofilm, and a large number of cocci covered the root canal dentin.

**3D Biofilm Structure of Dual Species**

A CLSM was used to scan the 3D biofilm structure of dual species and detect alive and dead cells. The biofilms of dual species of *E. faecalis* and *A. viscosus* (Fig. 4C) were significantly thicker than those of *E. faecalis* and *C. albicans* (Fig. 4A), *E. faecalis* and *L. acidophilus* (Fig. 4D), and *E. faecalis* alone (Fig. 4E), and there was a statistically significant difference between biofilm thickness of ES or EA group and the control group (Fig. 4F). (*P* < .05). In the group of *E. faecalis* and *C. albicans* (Fig. 4A) and *E. faecalis* and *A. viscosus* (Fig. 4C), some red dead cells were found.
cells were shown, and, in the other 3 groups, green viable cells dominated the biofilm.

**Discussion**

The pathogenic bacteria of root-filled canals with apical periodontitis are frequently restricted to a few gram-positive bacteria, but persistent infection is often caused by polymicrobial action. Although *E. faecalis* was frequently found in root-filled teeth with apical periodontitis, the strains were not isolated as a monoinfection in most cases (21, 22). Therefore, we explored the dual-species model of *E. faecalis* and the other 4 common species found in root-filled canals. To quantify either bacterium in dual species, 2 antibiotics, vancomycin and cefuroxime sodium, at final concentrations of 100 mg/L were added to the medium to distinguish them according to their different MIC values. The species of MIC <100 mg/L did not grow, and the bacterial colony on the plate agar was equal to the quantity of the other species.

The bacteria’s resistance to starvation may determine whether the bacteria survive in root-filled canals. The survival of a single bacteria, such as *E. faecalis*, *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius*, *Prevotella intermedia*, or *Pseudoramibacter alactolyticus* and a fungus (*C. albicans*), has been previously examined (23–25). The starvation endurance of dual species was evaluated in our studies, and we found that *E. faecalis* showed more resistance to starvation in the presence of *C. albicans*, *S. gordonii*, *A. viscosus*, and *L. acidophilus*, respectively, than alone. Under deficient nutrient conditions, the death and decomposition of *C. albicans*, *S. gordonii*, *A. viscosus*, or *L. acidophilus* might provide some essential ingredients for *E. faecalis* survival. Although the low nutrient composition does not meet the requirements of the growth of *E. faecalis*, it may in part help the resistance to starvation of *E. faecalis*. However, on the contrary, *S. gordonii* did not survive in the presence of *E. faecalis*. Nutrient deficiency may activate interspecies competition. *E. faecalis* is an *Enterococcus* spp, and it can produce bacteriocins, such as enterocin and cytolysin, which inhibit competitors in the microflora (26, 27). *S. gordonii* might be sensitive to the bacteriocins produced by *E. faecalis*, and, thus, it was inhibited in dual species. However, the reason that *C. albicans*, *A. viscosus*, and *L. acidophilus* have similar starvation kinetic curves in the presence and absence of *E. faecalis* might be because of the three microorganisms’ insensitivity to antibacterial agents produced by *E. faecalis*. This speculation needs to be examined further.

Multispecies biofilms of root canal bacteria have been described in previous studies (28, 29). *E. faecalis* biofilms on the biotic and abiotic surface are usually unstable and easily disturbed. The dual-species biofilm model of root canals showed that *E. faecalis* formed a dense biofilm structure on the root canal dentin in the presence of *S. gordonii* and *A. viscosus*, respectively. *S. gordonii* and *E. faecalis* in SEM were not distinguished because of their similar morphologies, but we discerned that *E. faecalis* dominated in the dual species of *E. faecalis* and *A. viscosus*, which was consistent with the results of Chávez de Paz et al (29), showing that *E. faecalis* OG1RF can inhibit Actinomyces...
naeslundii by using miniflow chambers and 16S ribosomal RNA filter in situ hybridization. A. viscosus and A. naeslundii belong to the same species and may have similar properties. Although S. gordonii and A. viscosus may promote E. faecalis growth, we have not yet observed dense biofilm on the root canal dentin in a 5-species model. From an ecological viewpoint, microorganisms maintain an ecological balance in microflora. E. faecalis may generate overpopulation in a dual-species model, but in a multispecies model, interaction between bacteria is very complex, and E. faecalis may keep a dynamic balance because of mutual competitive inhibition between bacteria. The aforementioned studies were performed with sufficient nutrition. However, abundant nutrition is not a normal state in root-filled canals. Under starvation conditions, S. gordonii was completely inhibited by OGI1RF, but A. viscosus showed better survival under starvation in coexistence with OGI1RF than alone, in contrast to the abundant nutrition in which A. naeslundii or A. viscosus was inhibited (29). E. faecalis was more resistant to starvation in coexistence with S. gordonii and A. viscosus than alone. These interspecific interactions may be why E. faecalis frequently dominates in root-filled canals.

Starvation and alkalinity are 2 important factors that influence the amount and species of microorganisms in the root canal system. Our studies indicated that E. faecalis was more resistant to starvation in coexistence with 4 other bacteria, and A. viscosus and S. gordonii promoted the survival of E. faecalis with abundant nutrition. The studies of van der Waal et al. (30) showed that calcium hydroxide favors the population of E. faecalis in dual-species biofilms in coexistence with Pseudomonas aeruginosa. These studies may help explain the prevalence of E. faecalis in persistent root canal infection; therefore, attention should be paid to the root canal bacteria that facilitate the overpopulation of E. faecalis and resistance to starvation with the microbiological factors of persistent root canal infection.

Acknowledgments

Yan Gao and Xiaoqiong Jiang contributed equally to this work. We are grateful to Elsevier Language Editing Services for professionally editing this article. Supported by grants from the Guangdong Natural Science Foundation (no. 2014A030313026). The authors deny any conflicts of interest related to this study.

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