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SYNERGISTIC GROWTH EFFECT AMONG BACTERIA RECOVERED FROM ROOT CANAL INFECTIONS

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ABSTRACT

The objective of this study was to determine the ecological relationships between bacterial species that colonize infected root canals. Root canal bacteria recovered from one patient with pulp canal necrosis were evaluated *in vitro* for synergistic and antagonistic activities determined by mono and co-culture growth kinetics and the production of bacteriocin-like substances using the double layer diffusion method. *Peptostreptococcus prevotii* triggered a significant increase of *Fusobacterium nucleatum* growth, while the former bacteria did not affect the growth of *P. prevotii*. The bacterial species did not produce antagonism activity against itself or against any of the other two species. Despite many studies have demonstrated the capability of root canal microorganisms to produce antagonistic substances, these *in vitro* experimental tests show the synergistic effect of *P. prevotii* on the growth of *F. nucleatum*.

Keywords: Root canal, infection, bacterial relationships.

INTRODUCTION

Microorganisms that inhabit the oral cavity may infect necrotic root canal systems (RCS) and induce inflammatory processes in adjacent periapical tissues. The microorganisms present in these infections belong to a limited group when compared to the potential candidates present in the oral cavity (18, 19), suggesting that some species are more adapted than others to colonize these sites (29, 30). There is a predominance of anaerobic species in these infections, and the majority of them can also be recovered from the periodontal pockets (36). Significant differences have been observed in the composition of pathogenic microbiota present in symptomatic and asymptomatic cases, suggesting that the structure of the bacterial community might determine clinical symptomatology (5, 25, 26, 35). Other researches did not find differences in the composition of the microbiota when comparing clinical symptomatology (2, 15).

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To date, although many studies have characterized the diversity of this microbiota, few of them have been focused on ecological relationships. Factors such as the oxygen balance, temperature, availability of nutrients, and bacterial interactions appear to influence the dynamic growth and colonization of the infected RCS (5, 6, 28, 29, 30).

Microbial interactions, which can be cooperative or interfering, frequently defined as synergism or antagonism relationships, have long been recognized as important determinants of ecological niches. Interspecies cooperation occurs by hydrolysis of complex carbohydrates and sequential fermentation of the resulting sugars, as well as by the exchange of growth factors. Common interference mechanisms include the competition for nutrients and adhesion sites, as well as the production of toxic metabolites and specific antimicrobial compounds, such as bacteriocins. Bacteriocins are bactericide proteins once thought to be effective only among closely related strains, but broader activity and selective action against distant relatives also occurs (33). Additionally, they may influence the organization of the biofilm that forms inside the infected RCS by inhibiting the growth of some species that compete for the same ecological niche (16, 27). Bacteriocin produced by strains of *Lactococcus lactis* has been used as a food preservative and evaluated as an antimicrobial agent to control dental plaque (13) as well as an endodontic antimicrobial solution (34). Although still relatively few characterized, it is known that bacteriocins are produced by oral microorganisms.

As the previous literature has rarely assessed microbial interactions in RCS infections, the present study attempted to determine possible ecological factors involved in microbial root canal colonization. We assayed synergistic and antagonistic relationships among strains recovered from one patient with pulpal necrosis (15) as well as their capability to produce bacteriocin-like substances.

MATERIALS AND METHODS

Bacterial strains

The microorganisms used in this investigation were recovered from one patient with necrotic pulp infection treated at the Endodontic Clinic of the Dental School at the Universidade Federal de Minas Gerais (15). The following microbial species were selected: *Fusobacterium nucleatum, Lactobacillus paracasei* and *Peptostreptococcus prevotii.*

"In vitro" assay for production of antagonistic substances

The selected species *L. paracasei, F. nucleatum* and *P. prevotii* were tested for its inhibition activity against itself as well as against the other species (*Clostridium butyricum*, *Prevotella intermedia*, *Streptococcus anginosus*, and *Gemella morbillorum*) also recovered from human necrotic pulp infection (15), using the double layer method (31). The *F. nucleatum* and *P. prevotii* strains were grown in BHI-S (Difco) and *L. paracasei* in de Man, Rogosa and Sharp broth (MRS, Difco), and incubated at 37ºC, for 24 hours (*L. paracasei*) and 48 hours (*P. prevotti* and *F. nucleatum*) in an anaerobic chamber (Forma Scientific Company, Marietta, OH, USA), containing an atmosphere of N2 85%, H2 10% and CO2 5%. Then, spots of 5 µl of the cultures were made onto BHI-S agar. The plates were incubated for periods of 24 hours and 48 hours, respectively, under the same anaerobic conditions. After incubation the plates were removed from the anaerobic chamber, exposed to chloroform vapor for 30 minutes and left open inside a laminar flow chamber for an equal period of time to allow evaporation of residual Chloroform. Plates were covered with 3.5 ml of soft agar (0.75% BHI-S or 0.75% MRS according to indicator species), inoculated with approximately 10^7 colony forming units (CFU) of the indicator bacteria and incubated under the specific cultivation conditions for each one. The presence of an inhibition zone around the spot indicated the production of antagonist substances. Diameter of the inhibition halo was measured using a digital pachymeter (Mitutoyo Sul América Ltda, Suzano, SP, Brazil). Experiments were done in duplicate.

Growth Curve Assay for the determination of bacterial synergistic relationship.

To ascertain *in vitro* synergism or antagonistic relationships between the microorganisms, individual and associated growth curves were determined for *L. paracasei, F. nucleatum* and *P. prevotii*. Associated growth curves were

established analyzing the three isolates in pairs. For inoculum standardization, individual growth curve of each microorganism were previously determined by measuring absorbance at different times of incubation using a spectrophotometer (Spectrum series SP-2100, Hangzhou, China) at 600 nm and by evaluating the number of Colony Forming Unit (CFU) per ml, to find the time range for logarithmic phase of each microorganism. Individual and co-cultures were then carried out using as inocula similar populations in their log phase as previously determined, to evaluate neutral, synergistic or antagonistic relationships, comparing the growth curve profile of each bacterium in pure culture with that obtained when co-cultured. Pure cultures were plated onto brain heart infusion (Difco, Sparks, MD, USA) agar supplemented with hemin and menadione (S-BHA), without antibiotics (control experiments). Organisms were co-cultured in supplemented BHI (S-BHI) and subsequently evaluated in respect to growth stimuli by plating to obtain bacterial counts onto S-BHI agar (S-BHA) containing an antibiotic selected to inhibit the growth of only one of the two microorganisms. For *L. paracasei-P. prevotii* co-culture, metronidazol (Sigma, St. Louis, LO, USA) was added to the S-BHA at a concentration of 1 µg/ml for selectively count of *L. paracasei*. For *L. paracasei-F. nucleatum* co-culture, amoxicillin trihydrate (Glaxo SmithKline, Rio de Janeiro, Brazil) and potassium clavulanate (Glaxo SmithKline) at 0.06 µg/ml were supplemented to BHI-S agar for *L. paracasei* count. For *P. prevotii- F. nucleatum* co-culture, erythromycin (Sigma) and chloramphenicol (Inlab, São Paulo, SP, Brazil) were added to the plate at 0.06 µg/ml to inhibit *F. nucleatum* and *P. prevotii* growth, respectively. Cultures were incubated in an anaerobic chamber containing an atmosphere of N_2, 10% H_2 and 5% CO_2 (Forma Scientific Inc., Marietta, OH, USA) at 37°C. At different periods of time culture samples (pure and associated) were removed and submitted to successive decimal dilutions. Cell growth was determined by counting the number of CFU/ml until 72 hours of culture.

Statistical analysis

Data were subjected to the normalization test (Shapiro-Wilk) and, subsequently, they were analyzed using an unpaired (Student's *t* test) test ($p < 0.05$).

RESULTS

Antagonistic assay

The selected bacterial species *F. nucleatum* and *P. prevotii* did not produce antagonism activity against itself or against any of the bacteria tested. *L. paracasei* inhibited the growth of *S.anginosus* and *G. morbillorum* (Table 1).

Table 1. Cross-test for presence (+) of bacteriocin-like activities between the selected bacteria.

Producing Strain	Target Strain						
	A	**B**	**C**	**D***	**E***	**F***	**G***
P. prevotii (A)	_	_	_	_	_	_	_
*F. nucleatum (*B)	_	_	_	_	_	_	_
L. paracasei (C)	_	_	_	_	_	+	+

*D- *C. butyricum*, E - *P. intermedia*, F - *S. anginosus*, G - *G. morbillorum*
+ Presence of antagonism; - absence of antagonism

Growth curves assay

In pure culture *F. nucleatum* showed a log phase occurring in the period of 12-48 hours of incubation and reaching a final cell density of 10^7 CFU/ml (Figure 1A). For *P. prevotii* a maximal cell density of about 10^8 CFU /ml was observed after 36 hours of incubation (Figure 1B). A short log phase (between 0 to 12 hours of incubation) was detected for *L. paracasei,* as can be seen in Figure 2.

The presence of *P. prevotii* triggered a significant increase in the growth of *F. nucleatum* after 60 hours of incubation (Fig. 1A, $p<0.05$). Therefore, co-culture did not affect the growth of *P. prevotii*, as demonstrated in Fig 1B ($p>0.05$).

Regarding the co-culture of *L. paracasei* and *F. nucleatum* (Fig. 2A), no statistically significant difference for growth of *L. paracasei* ($p>0.05$) was observed when compared to its pure

culture. The influence of *L. paracasei* on the growth of *F. nucleatum* was not evaluated in this study because no antibiotic concentration could be found that inhibited *L. paracasei* without influencing *F. nucleatum*.

Co-culture of *L. paracasei* and *P. prevotii* also did not show significant influence for *L. paracasasei* growth ($p<0.05$) when compared to the pure culture (Fig. 2B), despite some statistically difference found in the graphic. Due to the inability to find a suitable antibiotic concentration, the effect of *P. prevotii* on *L. paracasei* was not assessed.

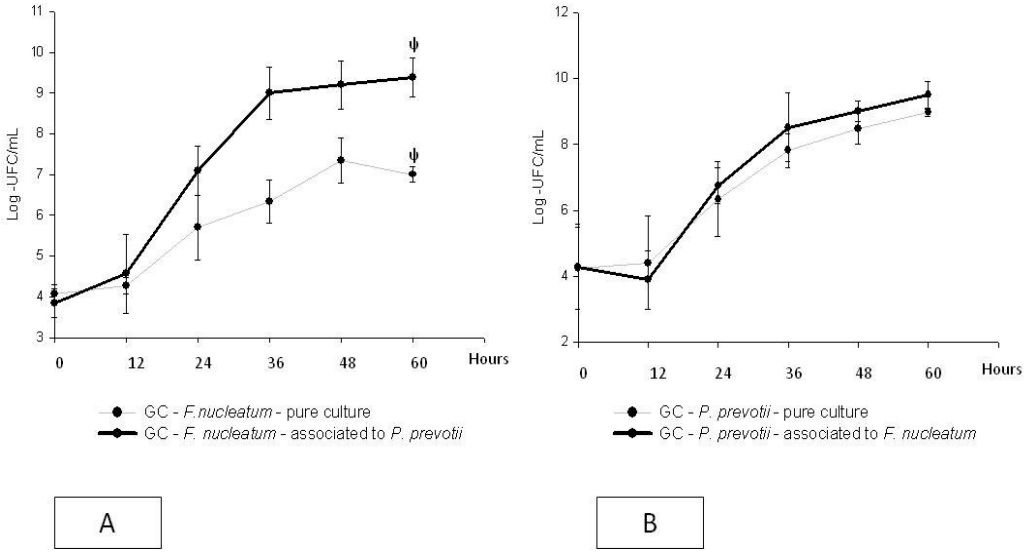

Figure 1. (A) Growth curve for co-culture between *F. nucleatum* and *P. prevotii*; (B) Growth curves for co-culture between *P. prevotii* and *F. nucleatum*. Comparisons between mono- and co-cultures were carried out as described in the Materials and Methods. Bars represent the means of three experiments, performed in duplicate, whereas the lines represent the standard errors of the mean. Ψ: statistical difference (t-test) between mono and co-culture ($p<0.05$).

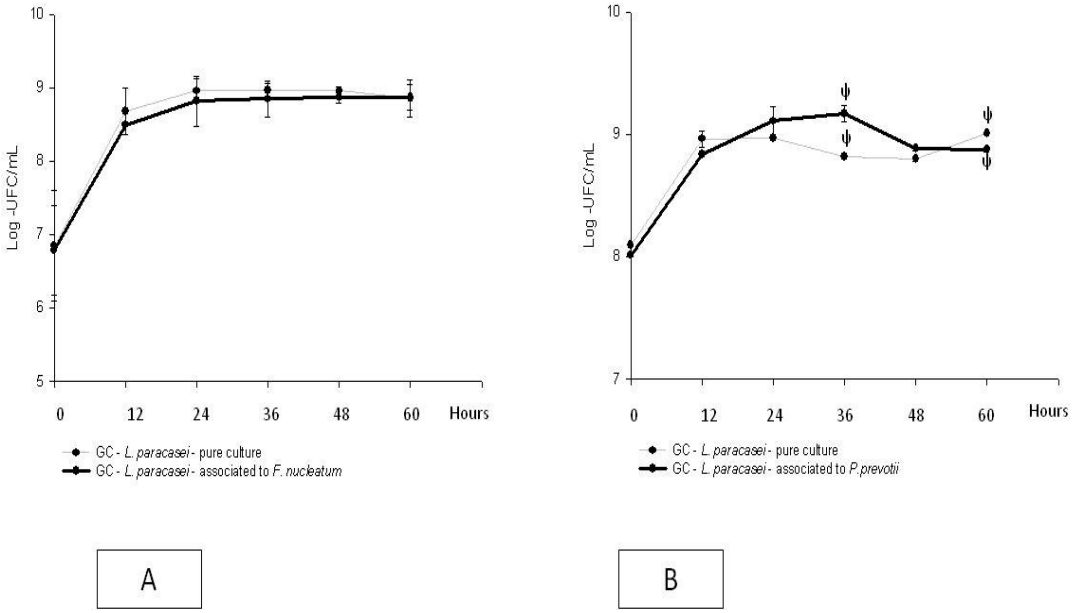

Figure 2. (A) Growth curve for co-culture between *L. paracasei* and *F. nucleatum*; (B) Growth curve for co-culture between *L. paracasei* and *P. prevotii*. Comparisons between mono- and co-cultures were carried out as described in the Materials and Methods. Bars represent the means of three experiments, performed in duplicate, whereas the lines represent the standard errors of the mean. Ψ: statistical difference (t-test) between mono and co-culture ($p<0.05$).

DISCUSSION

Microbial consortia play an important role in the etiology and pathogenesis of oral diseases, determining clinical parameters of inflammation and periodontal destruction (27, 28). Ecological factors such as synergistic and antagonistic interactions, co-aggregation, and availability of nutrients influence the composition of a given microbiota (10).

In root canal infections, specific bacterial species and combinations of species determine the composition of the microbial ecology, providing indispensable growth factors and physico-chemical changes in the environment (28, 29). Similarly, the evolution of clinical signs and symptoms as well as the destruction of apical bone can be influenced by relationships between bacterial species (9, 16, 18). The present study assessed synergism and antagonism among microorganisms recovered from human root canal infections (15) as well as their capability to produce bacteriocin-like substances. Control reference strains (ATCC) were not assayed in this study, since recent clinical isolates apparently express virulence factors different from those observed in culture collection strains (1). Additionally, to evaluate synergistic effects the growth stimuli is better furnished by strains isolated from the same infected site.

Microbial relationships were analyzed through the comparison of growth curves of microorganisms in mono or co-cultures. Previous investigators have found that specific bacterial combinations play a role in the evolution of infectious processes, particularly in pulp and periapical diseases (12, 32). The presence of organisms such as *Peptostreptococcus*, *Eubacterium*, *Fusobacterium*, and some black pigmented Gram negative strict anaerobic rods have been associated with symptomatic root canal infections (4, 7, 20). Conversely, facultative anaerobes such as *Streptococcus* and enteric bacteria have been linked to asymptomatic infections (4, 12).

The results presented here showed that the growth of *P. prevotii* (Figure 1B) and *L. paracasei* (Figure 2A and 2B) were not altered by co-culture with *F. nucleatum* or *P. prevotti,* respectively. On the other hand, the synergistic effect of *P. prevotii* on the growth of *F. nucleatum* was demonstrated by the significant growth increase of *F. nucleatum* ($p<0.05$), (Figure 1A) when co-cultured. Although many studies have demonstrated the capability of root canal microorganisms to produce antagonistic substances (4, 8, 22), this is the first study to show synergistic effect between *Fusobacterium* and *Peptostreptococcus*.

Several studies have been assayed bacterial antagonistic activity in root canal microbiota (3, 21). According to Klaenhammer (14), lactic acid bacteria produce antibacterial substances that vary in their spectrum of activity, mode of action, molecular mass, genetic determinants, and biochemical characteristics. Their actions can be auto - or hetero-antagonistic and affect a wide variety of targets including Gram-negative bacteria (17). In this study, *L. paracasei* presented antagonistic activity against *S. anginosus* and *G. morbillorum*.

Ribeiro-Sobrinho *et al.* (22) reported strong autoantagonistic activity of *F. nucleatum* and against *G. morbillorum.* In the present study, *F. nucleatum* presented neither auto- nor hetero-antagonism toward tested bacteria. Oliveira *et al.* (16) also did not detect antagonistic activity of *F. nucleatum* toward *P. intermedia, P. nigrescens* or *P. gingivalis*. In a previous study, *F. nucleatum* recovered from the same infection (15) was shown to survive and translocate to the sub-mandibular lymph node of germ-free mice (22), to modulate the periapical response through the expression of a particular profile of cytokines in murine root canal infections (23), and to induce apoptosis in murine lymph node cells (24).

Most previous studies examining the ecological complexity of root canal infections have shown antagonistic bacterial activity in these sites (2, 3, 16, 21). The present study shows that synergism could also occur. However, these potential mechanisms described here and in all literature have been demonstrated by *in vitro* assays and such result can not be always extrapolated to *in vivo* environment. Further comprehensive microbiological studies of multiple samples and

under *in vivo* conditions are necessary to better understand the ecological determinants that affect these infections, thus culminating in the development of new prevention and treatment procedures.

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