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Antagonist effect of probiotic bifidobacteria on biofilms of pathogens associated with periodontal disease

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ABSTRACT

The *in vitro* antagonist growth effect of bifidobacteria were evaluated on periodontal bacteria. *Bifidobacterium longum*, *Bifidobacterium lactis* and *Bifidobacterium infantis* biofilms were grown in single, double or triple combinations with putative periodontal pathogens *P. gingivalis* and *F. nucleatum* or beneficial bacteria *S. oralis* for 24, 72 and 168 h and the total counts were analyzed by checkerboard DNA-DNA hybridization. The results showed that *B. infantis* and *B. lactis*, as single species, demonstrated the best antagonist effect on *F. nucleatum* and *P. gingivalis* and no influence on *S. oralis* growth at 168 h. All the double combinations of bifidobacteria tested demonstrated an inhibitory effect on *F. nucleatum* (72 h) and *P. gingivalis* (168 h) and did not affect *S. oralis* counts at any time. In conclusion, *B. lactis* and *B. infantis* alone or in double combinations have antagonist effect on periodontopathogens biofilms, at different time points, and minimal influence on *S. oralis* growth.

1. Introduction

Systemic or local antibiotic periodontal therapy is usually administered as an adjunctive for reducing or eliminating bacteria potentiating the effects of traditional periodontal mechanical therapy and preventing the recurrence of infection [1]. However, the use of systemic antibiotics has some disadvantages such as the risk of development of bacterial resistance, collateral drug reactions, patient compliance and reduced concentrations of these antimicrobial agents in the subgingival sites [2]. Some studies have demonstrated the *in vitro* and in vivo impact of probiotic such as *Lactobacillus* spp. on the reduction of caries-related bacteria [3,4] or putative periodontal pathogens [4–7] and the expression of their virulence factors such as exotoxins [8,9], fimbriae, capsules and quorum sensing components [9]. However, few studies have investigated the relationship between species of *Bifidobacterium* and periodontal pathogens [5,7–11]. Probiotic *Bifidobacterium animalis* subsp. *lactis* Bb12 and of oral *Bifidobacterium dentium* and *Bifidobacterium longum* isolates when combined with *Porphyromonas gingivalis*, *Actinomyces naeslundii*, *Fusobacterium nucleatum* in models of subgingival biofilms reduced significantly only the number of *P. gingivalis*, however, the association of bifidobacteria into supragingival biofilms with *Streptococcus mutans* and *A. naeslundii* was less efficient, and *S. mutans* was not affected by the presence of any of these probiotics [10].

This study aimed to explore the *in vitro* antagonist growth effect of some probiotic species of bifidobacteria, either alone or in combination, on biofilms of *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Streptococcus oralis* at different time points, using the checkerboard DNA-DNA hybridization technique.

2. Material and methods

Fusobacterium nucleatum subsp. *nucleatum* (ATCC 25585), *Porphyromonas gingivalis* (33277) and *Streptococcus oralis* were grown in media supplemented Brain Heart Infusion Agar (BHI) while *Bifidobacterium longum* subsp. *longum* (ATCC 15707), *Bifidobacterium longum* subsp. *infantis* (ATCC 15697) and *Bifidobacterium animalis* subsp. *lactis* (ATCC

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27673) were grown in MRS broth and L-cysteine [11].

Single, double or triple combinations of *Bifidobacterium longum*, *Bifidobacterium lactis* and *Bifidobacterium infantis* were grown in biofilms with *P. gingivalis*, *F. nucleatum* or *S. oralis*. Bacterial strains were added at 10^8 cells/mL in equal volumes to 12-well plates and incubated for 24, 72 and 168 h at 37 °C. All experiments were performed in triplicate, in three different days. After these periods, bacterial pellets were lysed and DNA from those cultures was isolated for posterior quantification using the checkerboard DNA-DNA hybridization, as described by Almaguer-Flores et al. [11]. This molecular identification method is able to differentiate precisely species of the same group (e. g. *Bifidobacterium* spp., as proposed by the present study) and periodontal pathogens because of the specificity of the probes [12–15]. Cross-reactions between the probes were checked prior to the study.

The absolute counts of each bacterium in combination were transformed into percentages of growth, based on the total number of bacteria, which grew alone (considered as 100% of growth), because of the different ranges of values obtained by the species studied [11]. All results were submitted to statistical analysis at 5% significance using ANOVA and Tukey tests.

3. Results

In the current study, the percentages of *F. nucleatum*, *P. gingivalis* and *S. oralis* combined with *B. longum, B. lactis or B. infantis* are shown in Fig. 1A, B and 1C, respectively. *B. lactis* and *B. infantis* significantly inhibited the *in vitro* growth of *F. nucleatum* after 24 h (27.8%; 2.1%, respectively), 72 h (17.2%; 1.25%) and 168 h (64.9%; 54%) of incubation. *B. longum* had a similar inhibitory effect (14.5%) on *F. nucleatum* in comparison with the other bifidobacteria after 72 h of growth. *B. longum, B. lactis and B. infantis* inhibited *P. gingivalis* only after 168 h of growth (58.9%, 61.3%, 27.1%). The growth of *S. oralis*, which is considered as beneficial bacteria related to periodontal health [9], was affected by bifidobacteria at 24 and 72 h. No bifidobacteria interfered in *S. oralis* growth after 168 h.

The percentages of *F. nucleatum, P. gingivalis* and *S. oralis* in double or triple combination with the bifidobacteria species are presented in Fig. 2A, B and 2C. After 24 h, no combination had an effect on the growth of *F. nucleatum*, except for the triple combination *B. longum* + *B. lactis* + *B. infantis* (11.3%). In contrast, all combinations affected the growth of *F. nucleatum* after 72 h (18.4–51.6%). After 168 h, *F. nucleatum* growth was not influenced by any combination of bifidobacteria. For *P. gingivalis,* only the combinations *B. longum* + *B. lactis* (41.8%) and *B. longum* + *B. lactis* + *B. infantis* (50.1%) inhibited growth after 72 h. All combinations significantly reduced the growth of *P. gingivalis* after 168 h (16–23.2%). None of the combinations affected the growth of *S. oralis,* except for the triple combination of bifidobacteria after 24 and 72 h.

4. Discussion

In this study, bifidobacteria demonstrated an inhibitory effect against periodontal pathogens at the time points evaluated. *B. infantis* and *B. lactis* demonstrated the best effect against *F. nucleatum* and *P. gingivalis* and minimally influenced the growth of *S. oralis*. The relationship between *B. infantis* and periodontal bacteria has been investigated by Haukioja et al. [5] who showed co-adherence between *B. infantis* and *F. nucleatum,* proving that these species could persist in subgingival sites colonized by *F. nucleatum.* Previous study has showed that levels of *P. gingivalis* were also reduced in the presence of the probiotic *Bifidobacterium animalis* subsp. *lactis* Bb12, oral *Bifidobacterium dentium* and *Bifidobacterium longum* isolates in models of subgingival biofilms [10]. Although no study was found showing the antagonist effect of *B. infantis* on periodontal pathogens, the inhibitory effect of this bifidobacteria on enteropathogens is well-known, interfering in the adherence of these Gram-negative bacteria to intestinal cells [16].

This current study showed that all the double combinations of bifidobacteria tested had an inhibitory effect against *F. nucleatum* (after 72 h) and *P. gingivalis* (at 168 h) and did not affect *S. oralis* growth. However, no study was found for the combinations of various species of *Bifidobacterium*. Considering the effects on periodontal health, the combination of *L. rhamnosus* and *B. animalis* subsp. *lactis* BB-12 demonstrated significant reductions in clinical parameters, without affecting the composition of the oral microbiota [4]. Probiotics could inhibit pathogens due to their ability to produce lactic acid, hydrogen peroxide, bacteriocins or other antimicrobial substances either alone or in combination [17]. Cell-free supernatants of *Lactobacillus* ssp. and *Bifidobacterium* ssp. were tested in multispecies biofilms with *P. gingivalis*, *S. oralis* and *Streptococcus gordonii*. They downregulated the expression of virulence factors genes encoding fimbriae (*mfa1, fimA*), proteases (*fsH, kgp rgpA*) and quorum sensing components (*luxS*) of *P. gingivalis,* notably *L. acidophilus* LA5 [9]. A reduction of vitamin K concentration and inhibition of *P. gingivalis* growth occurred in the presence of *B. adolescentis* and *B. longum*. Both bifidobacteria and *P. gingivalis* require vitamin K for their growth and probably compete for its acquisition in the oral cavity [6]. Acids organics such as lactic acid produced by bifidobacteria causes damage to Gram-negative bacteria by disrupting the outer membrane or by its chelating capacity [18].

In a clinical practice guideline, European Federation of Periodontology (EFP) could not confirm that adjunctive agents, such a probiotics, are effective in controlling gingival inflammation because there is no adequate evidence to support their indication [19]. Based on this information, new studies should be conducted evaluating the efficacy of different species of probiotics, such as bifidobacteria, in helping to control pathogenic biofilm formation and to be useful in supportive periodontal care. Although *in vitro* biofilm models have their limitations and cannot completely reproduce the complexity of the oral environment; they have some advantages such as the absence of ethical conflicts, besides to analyze a variety of important in vivo interferences in a highly reproducible *in vitro* manner [20].

5. Conclusion

This study concluded that *B. lactis* and *B. infantis,* alone or in double combinations, can cause an antagonistic effect toward periodontopathogens and could be useful as coadjutants in periodontal therapy.

Author statement

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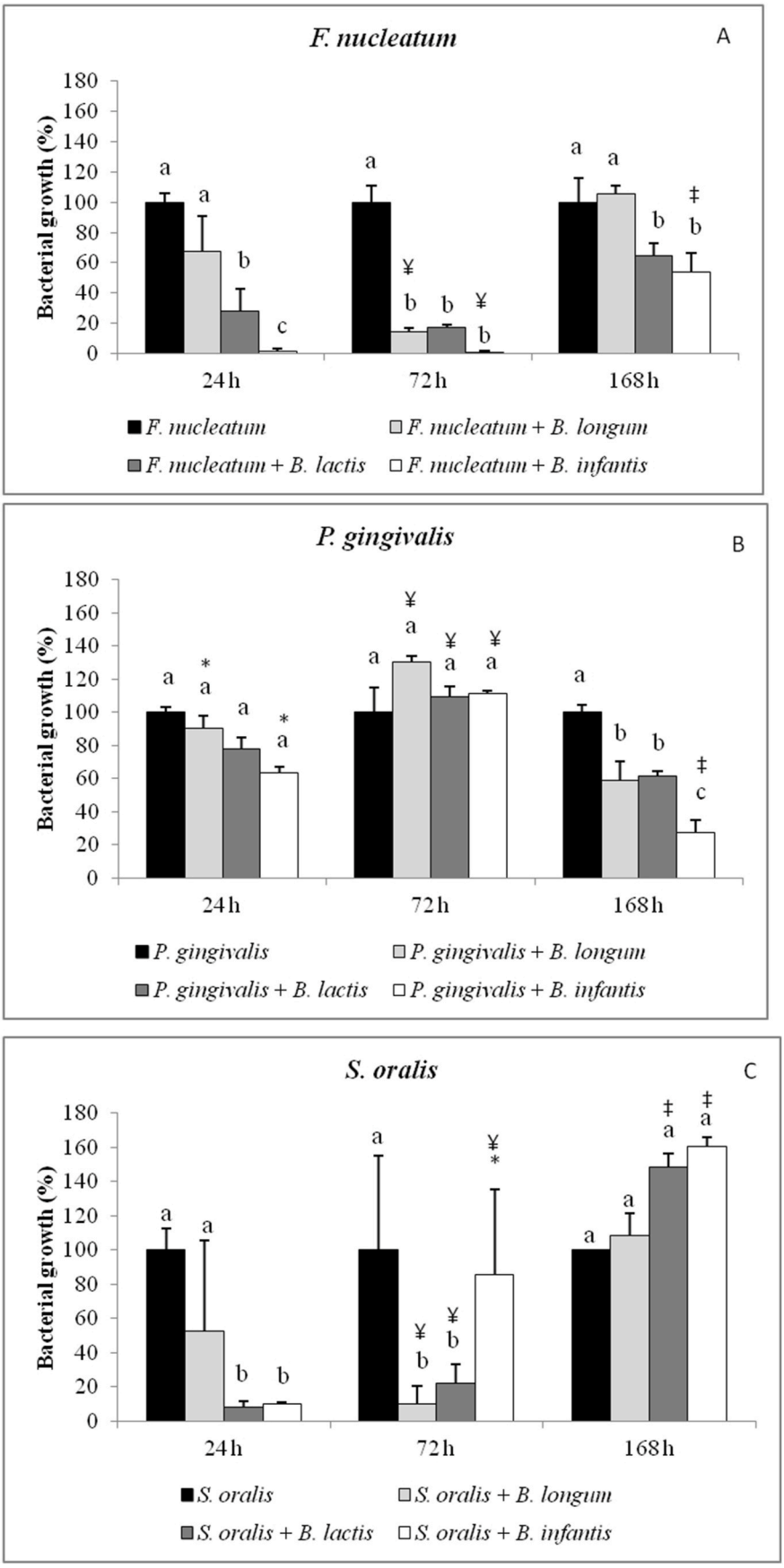

Fig. 1. Antagonism of single species of bifidobacteria on *F. nucleatum* (A), *P. gingivalis* (B) and *S. oralis* (C) biofilms. [a] Different lowercase letters show statistical difference among the groups of bifidobacteria, according to ANOVA and Tukey tests. * Statistical difference between 24 and 72 h, considering each group separately, according to ANOVA and Tukey tests. ‡Statistical difference between 24 and 168 h, considering each group separately, according to ANOVA and Tukey tests. ¥ Statistical difference between 72 and 168 h, considering each group separately, according to ANOVA and Tukey tests.

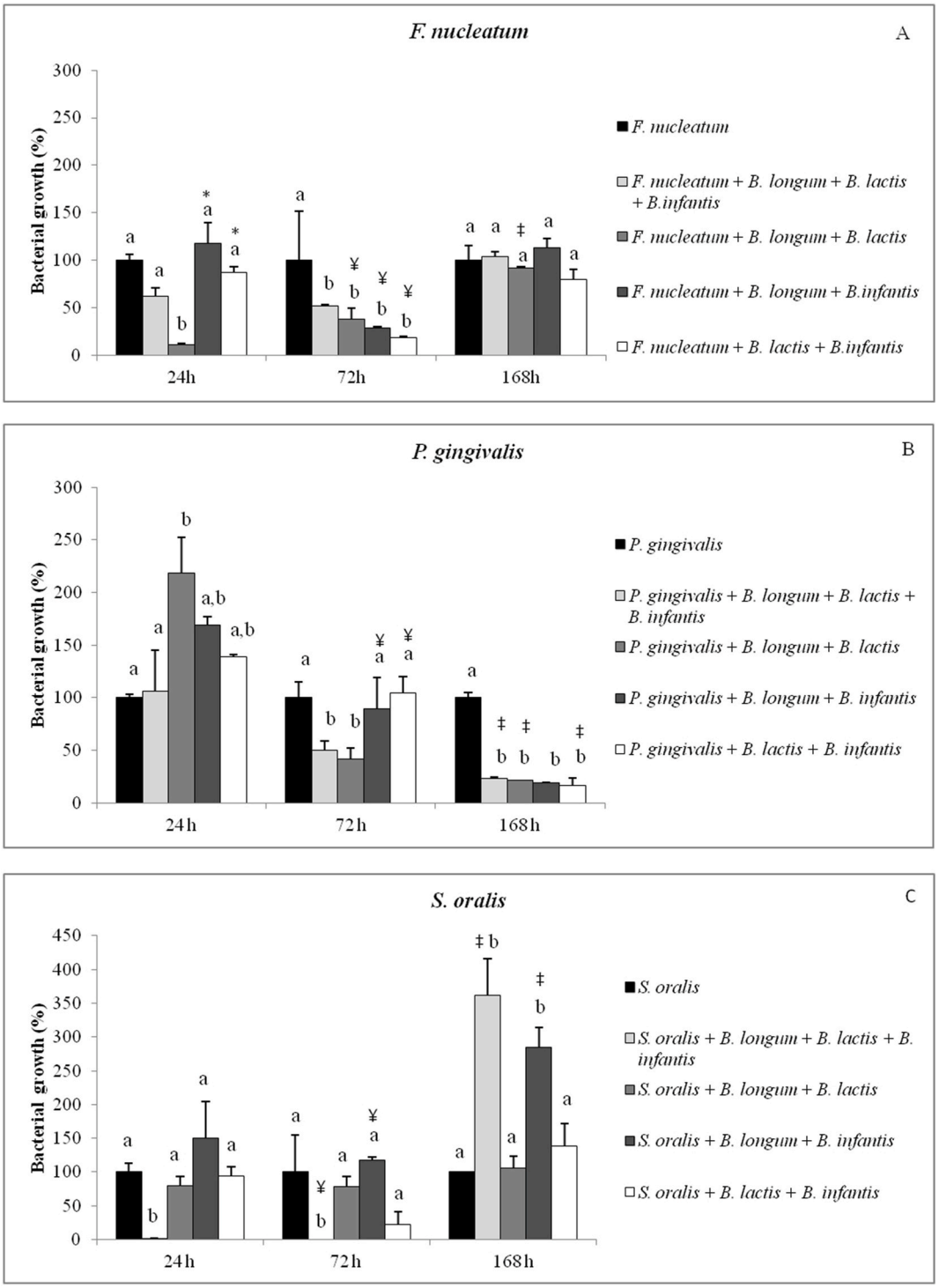

Fig. 2. Antagonism of the combinations of bifidobacteria on *F. nucleatum* (A), *P. gingivalis* (B) and *S. oralis* (C) biofilms.
[a] Different lowercase letters show statistical difference among the groups of bifidobacteria, according to ANOVA and Tukey tests. * Statistical difference between 24 and 72 h, considering each group separately, according to ANOVA and Tukey tests. ‡Statistical difference between 24 and 168 h, considering each group separately, according to ANOVA and Tukey tests. ¥ Statistical difference between 72 and 168 h, considering each group separately, according to ANOVA and Tukey tests.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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