Short Communication
Cariology

Effect of S. mutans combinations with bifidobacteria/lactobacilli on biofilm and enamel demineralization

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Abstract: The present study evaluated the ability of *Bifidobacterium* and *Lactobacillus* species associated with streptococci to increase insoluble extracellular polysaccharide (EPS) production and initial caries lesion progression. Bovine enamel blocks (n = 190; 4 mm x 4 mm) were prepared, selected according to initial surface hardness (SH), and divided into two groups: a) double combinations: *S. mutans* with *Bifidobacterium* or *Lactobacillus*, and b) triple combinations: *S. mutans* and *S. sobrinus* with *Bifidobacterium* or *Lactobacillus* species. The blocks were exposed to the bacterial associations for 7 days. Subsequently, quantity of EPS from biofilms and caries lesion depth were determined by means of colorimetric and cross-sectional enamel hardness (ΔKHN) analysis. The data were submitted to one-way analysis of variance, followed by the Bonferroni test ($p < 0.05$). *S. mutans* with *B. animalis* or *B. dentium* produced a higher quantity of EPS; *S. mutans* + *B. animalis* led to the highest ΔKHN. *S. mutans* + *S. sobrinus* + *B. longum* induced greater EPS and ΔKHN values. In conclusion, associations of *B. animalis* and *B. longum* with streptococci promoted EPS production and caries lesion progression.

Keywords: *Bifidobacterium*; *Lactobacillus*; *Streptococcus*; Polysaccharides; Dental Caries.

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Introduction

Frequent dietary carbohydrate intake can cause dysbiosis of the microbial community, triggered by the overproduction of acids by bacteria. This excessive acid load causes demineralization of the hard tissues of the tooth and development of dental caries.[1,2] Mutans streptococci, especially *Streptococcus mutans,* are still considered the most cariogenic bacterial group.[3] However, other acidogenic and acid-tolerant species may be involved in the onset and progression of caries lesions.[2,4] Species of the genus *Bifidobacterium,* also known as bifidobacteria, have received much attention, owing to their beneficial role in human health, including increased adaptive immune response, treatment or prevention of respiratory and urogenital tract infections, and prevention of allergies and atopic diseases in childhood, which is why they are included in food products.[5] However, studies have detected *Lactobacillus* and *Bifidobacterium* in biofilms of white spot lesions and dentin caries.[6] In a prior study, *B. animalis and*

B. longum were the most acidogenic and aciduric strains, comparable to caries-associated bacteria, such as *S. mutans* and *L. casei*.[7]

S. mutans is able to produce enzymes called glucosyltransferases, which hydrolyze sucrose from the diet into glucose and fructose. Glucose residues bind to each other to form extracellular polysaccharides (EPS) or insoluble glucans, responsible for adhesion of microorganisms to dental surfaces, and formation of the extracellular matrix that structures the dental biofilm. Biofilm formation results from sugar degradation and so-called microbial coaggregation, a process whereby genetically distinct microorganisms specifically recognize and become attached to one another. This interaction promotes organizational and cell-cell interactions that increase the resistance of the species individually, and the biofilm as a whole.[8] A previous study found a significant increase in biofilm formation and enamel demineralization when *S. mutans* were combined with *B. animalis* and *B. longum*.[7] Thus, this study proposed researching a sequence to this earlier study, involving the ability of some species of *Bifidobacterium* and *Lactobacillus* to combine with streptococci, and ultimately increase insoluble EPS production, leading to initial caries lesion progression.

Methodology

Bacterial strains and growth conditions

The bacterial species evaluated in this study were *B. animalis* (from ACTIVIA®), *B. longum*, (ATCC 15707), *B. lactis* (LMG 18905), and *B. dentium* (ATCC 27678); *L. acidophilus* (ATCC 4356), *L. casei* (ATCC 393); *S. mutans* (ATCC 25175 and 3VF2), and *S. sobrinus* (ATCC 27607). All ATCC strains were obtained from the Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, RJ, Brazil), and the André Tosello Foundation (Campinas, SP, Brazil). *S. mutans* 3VF2 is a highly acidogenic clinical strain that was previously characterized and also provided by Dr. Renata de Oliveira Mattos-Graner (FOP-UNICAMP, Piracicaba, Brazil).[9] *B. animalis* was isolated from ACTIVIA® yogurt. *Bifidobacterium*, *Lactobacillus,* and *Streptococcus* species were isolated from the following media: transgalactosylated oligosaccharides propionate agar supplemented with lithium mupirocin (50 mg/L) (TOS-MUP agar; Merck Millipore, Darmstadt, Germany), Rogosa Agar (Difco Laboratories, Detroit, USA) and Mitis Salivarius Agar (MSA, Difco), respectively.[7] All bacteria were grown at 37°C for 48 h in a 5% CO_2 atmosphere.

Experimental design

After approval of the Ethics Committee of the Araçatuba Dental School-UNESP (number: 197/2013), enamel blocks (4 mm × 4 mm, n=190) were obtained from bovine incisors, and kept in 2% formalin, pH 7.0, for 30 days.[10] The enamel surface of the blocks was polished, after which the initial surface hardness (SHi) was measured using a 5114 MicroMet microhardness tester (Buehler, Lake Bluff, IL, USA) with a Knoop-type indenter, and with a static load of 25 g for 10 s. Five indentations 100 µm apart were made in the central region of each block. The experimental design was randomized, and the blocks were divided into two or three bacterial combinations: Group 1 (n = 90): combinations with *Streptococcus mutans* (3FV2) totaling 9 combinations; Group 2 (n = 80): combinations with *S. mutans* + *S. sobrinus* totaling 8 combinations. Each group had *S. mutans* (n = 10) and *S. mutans* +*S. sobrinus* controls (n = 10), which were also analyzed.

In vitro initial caries lesion induction and enamel hardness analysis

The induction of artificial caries was modeled after the study by Lima et al.,[11] and modified by Valdez et al.[7] The bovine enamel blocks were completely isolated with a thin layer of nail varnish, except for the external surface (area = 16 mm^2), and placed individually into a modified artificial caries solution (brain heart infusion supplemented with 1% yeast extract, 0.5% glucose, 1% sucrose, and 2% of the bacterial culture – 10^8 cells/mL) for 7 days at 37°C. The culture medium was changed every 48 h. Bacterial viability was checked during the experiment after the dilution of random wells, and the plating of each type of bacteria in specific media (TOS-MUP agar for bifidobacteria, Rogosa Agar for lactobacilli, and MSA for streptococci).[7] Afterwards, 14 indentations were made in the enamel, at different distances – 5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm – and different depths from the surface, in the central region. The indentations were spaced

100 μm from each other, as measured with a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA) and the Buehler OmniMet software program (Buehler), and were made with a Knoop diamond indenter under a 5-g load for 10 s. The averages were calculated for each distance. The integrated hardness (KHN x μm) of the lesion was calculated by the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the integrated hardness for sound enamel, to obtain the integrated area of the subsurface regions in the enamel. This integrated area was named subsurface enamel hardness (ΔKHN; KHN x μm).[10]

Insoluble extracellular polysaccharide (EPS) analysis

After 7 days, the biofilm that formed on the enamel blocks was collected and analyzed.[12,13] Carbohydrate analysis was performed using the phenol-sulfuric acid procedure,[14] and the results were expressed in μg/g EPS (dry weight).

Statistical analysis

Statistical analysis was performed using the SPSS program version 17.1, considering $p < 0.05$ as significant. The data for DKHN and EPS were found to be normal (Shapiro-Wilk) and have homogeneous (Bartlett) distribution, and were analyzed using one-way analysis of variance (ANOVA), followed by the Bonferroni test.

Results

When *S. mutans* was inoculated with *L. casei shirota* and *B. longum*, ΔKHN was similar to *S. mutans* inoculated alone. In the other groups, there was a greater loss of hardness compared with the control group ($p < 0.05$). The double combination that induced the highest loss of subsurface hardness was *S. mutans* + *B. animalis* (Table 1). The *S. mutans* + *S. sobrinus* + *B. longum* group induced a greater loss of subsurface hardness compared with the *S. mutans* + *S. sobrinus* group, and the highest quantity of EPS compared to the other groups ($p < 0.05$) (Table 2).

Discussion

In the present study, the association of the same species of Bifidobacteria, *B. animalis* and *B. longum* with *S. mutans* or with *S. mutans* + *S. sobrinus*, respectively, induced the highest loss of subsurface enamel hardness. *Bifidobacterium* and *Lactobacillus* adhere poorly to the dental structure, and require mediation by other oral bacteria. Consequently, they are unable to form biofilm, as formed by cariogenic bacteria.[7] In line with the aims of the present article, dental enamel demineralization tests were performed with these species, together with *S. mutans* and/or *S. sobrinus*. Campos et al.[15] showed that the associations of *S. mutans* and *L. casei* or *S. mutans*, *L. acidophilus* and *L. casei* led to greater loss

Table 1. Mean (standard deviation) of subsurface enamel hardness (ΔKHN) and insoluble extracellular polysaccharides (EPS) measurements for the double combinations.

Group	ΔKHN (KHN x μm)	EPS (μg/g)
S. mutans	5,025.5 (2,401.1)^{A}	2.6 (0.9)^{a}
S. mutans + *S. sobrinus*	7,794.6 (1,202.8)^{B}	3.1 (1.1)^{a}
S. mutans + *L. casei*	6,690.4 (1,953.0)^{B}	2.8 (0.9)^{a}
S. mutans + *L. casei shirota*	4,984.0 (1,508.5)^{A}	2.3 (0.7)^{a}
S. mutans + *L. acidophilus*	7,079.6 (3,321.1)^{B}	5.5 (2.3)^{b}
S. mutans + *B. dentium*	7,281.7 (1,637.4)^{B}	4.5 (1.6)$^{b, c}$
S. mutans + *B. longum*	4,140.3 (1,613.3)^{A}	2.1 (0.6)^{a}
S. mutans + *B. animalis*	9,186.9 (1,859.7)^{C}	3.9 (1.6)^{c}
S. mutans + *B. lactis*	7,096.9 (2,970.0)^{B}	2.7 (0.7)^{a}

^{A}Different uppercase letters show statistical difference among the groups, according to the ANOVA and Bonferroni tests; ^{a}Different lowercase letters show statistical difference among the groups according to the ANOVA and Bonferroni tests.

Table 2. Mean (standard deviation) subsurface enamel hardness (ΔKHN) and insoluble extracellular polysaccharide (EPS) measurements for triple combinations.

Group	ΔKHN (KHN x μm)	EPS (μg/g)
S. m + S. sobrinus	7,794.6 (1,202.8)[A]	3.1 (1.1)[a]
S. m + S. s + L. casei	5,862.8 (3,850.3)[B]	4.7 (1.2)[b]
S. m + S. s + L. casei shirota	6,373.1 (2,511.2)[B]	5.1 (2.2)[b]
S. m + S. s + L. acidophilus	8,689.6 (1,504.7)[A]	4.4 (1.4)[b]
S. m + S. s + B. dentium	6,958.2 (1,819.4)[A]	5.3 (3.3)[b]
S. m + S. s + B. longum	10,022.3 (2,487.9)[C]	6.1 (2.3)[c]
S. m + S. s + B. animalis	8,040.5 (3,516.3)[A]	5.2 (0.8)[b]
S. m + S. s + B. lactis	5,563.9 (3,011.2)[B]	3.2 (1.7)[a]

[A]Different uppercase letters show statistical difference among the groups, according to ANOVA and Bonferroni tests; [a]Different lowercase letters show statistical difference among the groups, according to ANOVA and Bonferroni tests

of enamel surface hardness and higher depth values of carious lesions, observed in the first four days of induction, and lesions similar to erosion after 20 days of cariogenic challenge, thus corroborating the results obtained in the present study.

Among the species of Bifidobacteria evaluated in this study, only *B. dentium* was isolated in the oral cavity. *B. dentium* led to the loss of enamel subsurface hardness when associated with *S. mutans* and with both *S. mutans* and *S. sobrinus*. In a previous study performed by this research group, the association of *B. dentium* with species of streptococci did not produce greater surface loss compared with streptococci evaluated alone.[7] In addition, *B. dentium* did not stand out as an acid-producing or acid-resistant strain, in comparison with *S. mutans* and other cariogenic species.[7]

In the present study, EPS was quantified from biofilms developed on the surface of bovine enamel. This is why the combinations of bacterial species followed the same pattern as that observed in the subsurface hardness analysis. Therefore, the species of bifidobacteria were evaluated only in combination with *S. mutans* and *S. mutans* + *S. sobrinus*. Although bifidobacteria cannot adhere to enamel, they contribute toward increasing EPS production, and may constitute a substrate for associated cariogenic bacteria. In the present study, *B. animalis* and *B. longum*, associated respectively with *S. mutans* or with *S. mutans* + S. *sobrinus*, produced greater loss of subsurface hardness, and also presented the highest quantity of EPS. This suggests that these species could provide more substrate for cariogenic bacteria, consequently increasing the biofilm biomass.

Conclusion

The association of *B. animalis* and *B. longum* with *S. mutans* or *S. mutans* + *S. sobrinus* promotes the greatest hardness loss and extracellular polysaccharide production.

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