Effects of probiotic fermented milk on biofilms, oral microbiota, and enamel

Abstract: The aim of this study was to evaluate in vitro and in vivo the effects of 2 brands of probiotic fermented milk on biofilms, oral microbiota, and enamel. For the in situ experiment, ten volunteers wore palatine devices containing four blocks of bovine dental enamel over 3 phases, during which 20% sucrose solution, Yakult® (Treatment A), and Batavito® (Treatment B) were dropped on the enamel blocks. Salivary microbial counts were obtained and biofilm samples were analyzed after each phase. For the in vivo experiment, the same ten volunteers drunk Yakult® (Treatment C) and Batavito® (Treatment D) in two phases. Saliva samples were collected for microbial analysis after each phase. The in situ study showed that in comparison with Treatment A, Treatment B resulted in fewer total culturable anaerobes and facultative microorganisms in biofilms, higher final microhardness, lower percentage change in surface hardness, and smaller integrated subsurface enamel hardness. In the in vivo study, Treatment D resulted in a reduction in the counts of all microorganisms. The results suggested that the probiotic fermented milk Batavito®, but not Yakult®, reduced the amount of oral microorganisms and mineral loss in bovine enamel.

Keywords: Probiotics; Tooth Demineralization; Dental Caries; Streptococcus Mutans; Lactobacillus.

Introduction

Research on probiotics has progressed over the last 20 years; significant advances have been made in the selection and characterization of specific probiotic cultures as well as in the substantiation of health claims related to their consumption.1 Although the mechanism underlying probiotic activity in the oral cavity is not fully understood, it is usually considered to be a combination of local and systemic immune responses as well as non-immunological defense mechanisms.2 The principal health-promoting effects are ascribed to the ability of probiotics to enhance the intestinal mucosal immune defense and macrophage activity and elevate the numbers of killer cells, T cells, and interferons.3 In order to show effectiveness against oral infections, probiotic bacteria need to adhere to the oral mucosa and dental tissues as part of the biofilm and compete with dental pathogens.4 The most widely used species belong to the genera Lactobacillus and Bifidobacterium, because these organisms are already produced in the dairy industry and are very

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rarely implicated in infections in humans, although some species seem to play a role in the microbiology of dental caries.

The archetypical probiotic food is yogurt, and daily consumption of dairy products seems to be the most natural way to ingest probiotic bacteria. Another advantage is that milk products contain basic nutrients for growing children; they are also considered safe for the teeth and have possible beneficial effects on the salivary microbial composition and inhibition of caries development due to their natural casein, calcium, and phosphorous content.

However, the optimal vehicle for probiotic delivery is yet to be determined. Çaglar et al. examined the effects of probiotics ingested via 2 different non-dairy delivery systems on the levels of salivary mutans streptococci and lactobacilli. They found that the probiotic significantly reduced the streptococcal levels in comparison with the placebo regardless of the delivery system. Further, Montalto et al. studied the effects of oral administration of lactobacilli on the salivary lactobacillus count and found that oral administration of probiotic lactobacilli, irrespective of the delivery system, significantly increased the salivary lactobacillus count.

Recently, we evaluated the properties of some fermented milk brands and their effects on the inorganic composition of biofilms. Although all the products were milk-based beverages and contained fluoride, calcium, and phosphate, they promoted caries in bovine enamel blocks. In this context, the aim of the present study was to evaluate in situ and in vivo the effects of 2 brands of probiotic fermented milk on biofilm, oral microbiota, and enamel, and to evaluate the effect of short-term ingestion of these beverages on oral microbiota.

**Methodology**

**Selection**

The fermented milk brands used were Yakult® (Yakult S/A Indústria e Comércio, Lorena, Brazil) and Batavito® (BRF S.A., Carambeí, Brazil) (hereafter, fermented milk 1 and 2, respectively).

According to the manufacturers, Yakult® contains a single probiotic bacterial species, *Lactobacillus casei* Shirota, and Batavito® contains a combination of 3 probiotic bacteria (*Lactobacillus acidophilus*, *Bifidobacterium* sp., and *Lactobacillus paracasei*).

**Total and Reducing Sugar Analyses of Fermented Milk**

The levels of total and reducing sugars were determined using the methods described by Dubois et al. and Somogyi-Nelson, respectively.

**Enamel Block Preparation and Analysis**

Enamel bovine blocks were prepared, polished, and selected as described by Lodi et al. The baseline (SMHi) and final (SMHf) enamel surface microhardness measurements was performed. The percentage change in SMH (%SMH) was calculated as follows: %SMH = 100 (SMHf − SMHi)/SMHi.

**In Situ Experiment**

This experiment was approved by the local human ethics committee (FOA-UNESP, protocol #2008-01519). Ten healthy volunteers were selected on the basis of the criteria defined in previous studies. The in situ experiment involved a randomized double-blind crossover design performed in 3 phases with 20% sucrose solution (control treatment), fermented milk 1 (treatment A), and fermented milk 2 (treatment B). Each participant wore an acrylic palatal device. In each phase, participants applied 2 drops of 20% sucrose solution, fermented milk 1, or fermented milk 2 on the enamel blocks 8 times a day. Five minutes after the application, the device was reinserted into the mouth. After 14 days, biofilm samples were collected for microbial analysis. A washout period of 7 days was maintained between each phase. Only one dietary restriction was imposed on the patients; they were asked to avoid consuming other sources of probiotic bacteria. The participants were also instructed to remove the appliance while eating and performing oral hygiene procedures. In addition, they were not allowed to use any antimicrobial or fluoride products during the experiment.
**In Vivo Experiment**

The same volunteers participated in the *in vivo* experiment. It involved a randomized single-blind crossover design performed in 2 phases, after the *in situ* study, with fermented milk 1 (treatment C) and fermented milk 2 (treatment D). Each participant drank 80 g of the fermented milk daily for 14 days. The dietary restrictions and conditions for oral hygiene were the same as those in the *in situ* study. Saliva samples were collected at the beginning of the experiment and the end of each phase for microbial analysis.

**Biofilm Analysis**

At the end of the *in situ* experiment, the biofilm was harvested. Around 5 mg of each biofilm sample was resuspended in PBS (0.1 M, pH 7.2; 1 mL/mg biofilm). The suspensions were serially diluted in PBS and plated on brain heart infusion agar (HiMedia Laboratories, Mumbai, India), mitis salivarius agar (HiMedia Laboratories), mitis salivarius sucrose bacitracin agar (Sigma-Aldrich Co., St. Louis, USA), and Rogosa agar (HiMedia Laboratories) to determine the total cultivable anaerobe and facultative (TCAF), total streptococcal, mutans streptococcal, and lactobacillus colonies, respectively. The plates for the TCAF, total streptococci, and mutans streptococci were incubated at 37°C for 48 h in an anaerobic chamber; the lactobacillus plates were incubated aerobically at 37°C for 72 h. Colonies were counted with a colony counter.

The remaining biofilm was dried with phosphorus pentoxide (Vetec Química Fina Ltda., Duque de Caxias, Brazil) for 12 h at room temperature. Insoluble extracellular polysaccharides (EPS) were extracted. Carbohydrates were analyzed by using the phenol–sulfuric acid procedure. The results are expressed as mg/g dry weight.

**Saliva Analysis**

Saliva was collected using PBS as oral rinse. After 1 min of rinsing, the samples were collected and centrifuged for 10 min at 8,000 × g, the supernatant was discarded, and 2.5 mL PBS was added to the pellet. The suspensions were serially diluted in PBS and plated as described for the biofilm analysis. The results are expressed as CFU/mL saliva.

**Statistical Analysis**

Statistical analysis was carried out by using BioEstat Version 5.0 (*Instituto de Desenvolvimento Sustentável de Mamirauá*, Belém, Brazil). Data on CFUs were logarithmically transformed. The data were analyzed by using a single-factor ANOVA model. Multiple comparisons were conducted by using the Tukey or Bonferroni test when significant effects (p < 0.05) were detected. Data were analyzed by using Kruskal-Wallis one-way analysis when they were not normally distributed or when the variances were not equal. Data (means) from microorganisms in the saliva samples before and after each phase were compared with paired *t*-tests. The significance limit was set at 5%.

**Results**

**Total and Reducing Sugar Content**

The mean total sugar content of 20% sucrose, fermented milk 1, and fermented milk 2 was 155.67, 158.49, and 191.33 mg/mL, respectively. Further, the mean reducing sugar content was 0.15, 9.83, and 0.83 mg/mL, respectively. There were significant differences in the total and reducing sugar content of the three treatment agents.

**Enamel Hardness**

The means and SD values of the different enamel hardness variables are shown in Table 1. The SMHi was not significantly different among the treatment groups (p = 0.853). However, treatment B showed significant differences with respect to the SMHf, percentage of surface hardness change, and integrated loss of subsurface hardness (p < 0.05). No significant differences in these variables were observed between treatment A and the control treatment (p > 0.05).

**In Situ Findings**

Regarding the biofilm data, the TCAF levels were lower after treatment B than after treatment A and the control treatment, but no significant difference was observed between treatment B and the control treatment, and between treatment A and the control treatment. The treatment groups did not show significant differences in the total streptococcal levels and the levels of mutans...
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Furthermore, the EPS concentration did not differ significantly among the treatments ($p > 0.05$; Table 2). Both the control treatment and treatment A produced 13.84 mg/g dry biofilm, whereas treatment B produced 12.35 mg/g dry biofilm.

In the saliva analysis, no significant difference was noted between the baseline and the final microbial levels in each treatment (Table 3).

### In Vivo Findings

Treatment C did not significantly alter the concentrations of any microorganisms from the baseline values. However, the levels of all analyzed microorganisms decreased significantly after treatment D (Table 4).

### Discussion

In our *in vivo* experiment, commercially probiotic fermented milk samples were orally administered, enabling direct contact with oral tissues. Although fermented milk has not been developed for preventing dental caries by promoting changes in the oral microbiota, treatment D decreased the salivary counts of all investigated microorganisms after 2 weeks of ingestion. These results corroborate the findings of previous studies.$^{8,21,22,23}$

Some studies of the effects of lactobacillus-based probiotics on mutans streptococci reported significant reductions in the levels of salivary mutans streptococci immediately after terminating daily intake.$^{8,21,22,23}$ The post-treatment reductions were not directly dependent on the delivery vehicles, which included milk, cheese, yogurt, lozenges, and straws prepared with freeze-dried strains. Çaglar *et al.*$^8$ investigated whether slowly melting tablets facilitate a more thorough contact between the probiotic and the oral environment compared with a direct swallowing pattern from a straw. Both methods of administration equally reduced the prevalence of salivary mutans streptococci after 2 weeks of use.

Contrary findings were reported by Montalto *et al.*, who evaluated the administration of probiotic lactobacilli in liquids and capsules to determine the role of direct contact of probiotics with the oral tissues. Interestingly, both means of administration significantly increased the salivary lactobacillus counts, whereas the mutans streptococci levels were not significantly altered. Some studies have indicated that direct contact with oral tissues is not a prerequisite for probiotics to have a

### Table 1. Mean and standard deviation (SD) of the variables analyzed in the enamel surface.

<table>
<thead>
<tr>
<th>Variable (n = 10)</th>
<th>20% Sucrose</th>
<th>Treatment A</th>
<th>Treatment B</th>
<th>Significance ($p$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMHi</td>
<td>$377.1^{a}$ (0.7)</td>
<td>$377.3^{a}$ (0.4)</td>
<td>$377.2^{a}$ (0.4)</td>
<td>$= 0.853$</td>
</tr>
<tr>
<td>SMHf</td>
<td>$76.0^{a}$ (66.8)</td>
<td>$127.0^{a}$ (74.8)</td>
<td>$189.6^{b}$ (67.0)</td>
<td>$&lt; 0.05$</td>
</tr>
<tr>
<td>%SMHf</td>
<td>$-79.8^{a}$ (17.8)</td>
<td>$-66.3^{a}$ (19.8)</td>
<td>$-49.7^{a}$ (17.8)</td>
<td>$&lt; 0.05$</td>
</tr>
<tr>
<td>ΔKHN</td>
<td>$9961.9^{a}$ (1468.2)</td>
<td>$8940.9^{a}$ (2800.3)</td>
<td>$4063.5^{a}$ (1697.2)</td>
<td>$&lt; 0.05$</td>
</tr>
</tbody>
</table>

$^{a,b}$Means (SD) followed by distinct letters are significantly different for the variable.

1Initial microhardness (Knoop hardness).
2Final microhardness (Knoop hardness).
3Percentage of subsurface hardness change (Knoop hardness).
4Integrated loss of subsurface hardness (Knoop hardness).

### Table 2. Mean and standard deviation (SD) of microorganisms in the biofilm (log CFU/mg) after 14 days (in situ study).

<table>
<thead>
<tr>
<th>Variable (n = 10)</th>
<th>20% Sucrose</th>
<th>Treatment A</th>
<th>Treatment B</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCAF</td>
<td>$6.87^{a,b}$ (0.49)</td>
<td>$7.04^{a}$ (0.24)</td>
<td>$6.66^{a}$ (0.47)</td>
</tr>
<tr>
<td>Total streptococci</td>
<td>$5.73^{a}$ (0.53)</td>
<td>$5.81^{a}$ (0.87)</td>
<td>$5.71^{a}$ (0.65)</td>
</tr>
<tr>
<td>Mutans streptococci</td>
<td>$3.24^{a}$ (1.38)</td>
<td>$2.99^{a}$ (0.99)</td>
<td>$3.14^{a}$ (1.22)</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>$5.19^{a}$ (1.97)</td>
<td>$5.55^{a}$ (0.75)</td>
<td>$5.18^{a}$ (1.67)</td>
</tr>
</tbody>
</table>

$^{a,b}$Means (SD) followed by distinct letters are significantly different for the variable.

1Total cultivable anaerobes and facultative microorganisms.
beneficial effect; purely systemic administration of a probiotic could enhance lactobacillus proliferation in the oral cavity.8,9,22

The conflicting results for probiotic efficacy among previous studies are probably attributable to differences among strains of the same species.24 At present, most research is carried out with well-defined dairy-based live lactobacillus strains. In our study, fermented milk 1 contained a single probiotic bacterial species and fermented milk 2 contained a mix of 3 probiotic bacteria. The better results after the consumption of fermented milk 2 can be explained by the difference in their probiotic contents: simultaneous application of different probiotic bacteria can affect the balance of the oral ecosystem via possible additive, cumulative, or competitive modes of action.3

It should be noted that saliva samples may underestimate the true contents of oral biofilms.25 Moreover, biofilms are difficult to collect in vivo because of the need for oral hygiene restrictions during experimentation. Therefore, we also performed an in situ experiment to investigate the effects of probiotic fermented milk on biofilms. In the in situ experiment, treatment B lowered the concentrations of TCAF in biofilm compared with the treatment A. However, the concentrations of total streptococci, mutans streptococci, and lactobacilli were not significantly different among the treatments. This condition can be explained by the fact that the optimal dose required for pathogenic bacterial suppression was not used during the experiment because the participants merely dripped the fermented milk onto the enamel blocks. Lee and Salminen26 suggested an intake of 100 g containing 10^6-10^7 probiotic cells as the optimal dose for intestinal benefits; similarly, Çağlar et al.27 showed a significant reduction in the number of mutans streptococci with a fixed amount of probiotic bacteria (53 g ice cream with 1 \times 10^7 CFU/g ingested daily). Microorganism reduction was also achieved with 200 g of yogurt containing 2 \times 10^8 CFU/g in their former study.28

To evaluate changes in the enamel surface and its demineralization, we used the microhardness test in the in situ experiment. Treatment B significantly differed from the other treatments with respect to the final microhardness, percentage of surface hardness change, and integrated loss of subsurface hardness. Both products used in this study were milk-based and contained fluoride, calcium, and phosphate.10 Although these ions should confer a protective effect

### Table 3. Mean and standard deviation (SD) of microorganisms in saliva (log CFU/mL) at baseline and after 14 days (in situ study).

<table>
<thead>
<tr>
<th>Variable (n = 10)</th>
<th>20% Sucrose</th>
<th>Treatment A</th>
<th>Treatment B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>14 days</td>
<td>Baseline</td>
</tr>
<tr>
<td>TCAF1</td>
<td>6.80± (0.69)</td>
<td>6.42± (1.0)</td>
<td>6.96± (0.79)</td>
</tr>
<tr>
<td>Total streptococci</td>
<td>6.20± (0.72)</td>
<td>5.82± (0.89)</td>
<td>6.38± (0.80)</td>
</tr>
<tr>
<td>Mutans streptococci</td>
<td>3.26± (1.08)</td>
<td>3.36± (0.91)</td>
<td>3.67± (1.60)</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>4.06± (0.95)</td>
<td>3.90± (0.82)</td>
<td>4.09± (0.79)</td>
</tr>
</tbody>
</table>

### Table 4. Mean and standard deviation (SD) of microorganisms in saliva (log CFU/mL) at baseline and after 14 days (in vivo study).

<table>
<thead>
<tr>
<th>Variables (n = 10)</th>
<th>Treatment C</th>
<th>Treatment D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>14 days</td>
</tr>
<tr>
<td>TCAF1</td>
<td>6.74± (1.25)</td>
<td>6.70± (1.15)</td>
</tr>
<tr>
<td>Total streptococci</td>
<td>6.04± (0.97)</td>
<td>6.19± (1.10)</td>
</tr>
<tr>
<td>Mutans streptococci</td>
<td>3.48± (0.94)</td>
<td>3.74± (1.21)</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>3.11± (0.79)</td>
<td>3.57± (1.49)</td>
</tr>
</tbody>
</table>

*#Means (SD) followed by distinct letters are significantly different for the variable.

1Total cultivable anaerobes and facultative microorganisms.
on enamel, enamel demineralization occurred in both treatments, possibly because of the presence of sucrose.

Sucrose is considered the most cariogenic factor present in human diets because of its fermentability and ability to act as a substrate for the synthesis of polysaccharides in biofilm.\textsuperscript{29} To interpret the aforementioned results, we investigated the amounts of total and reducing sugars in both brands of fermented milk and the EPS concentration in biofilm. Both brands contained sugars; moreover, EPS was detected in biofilm, which could explain the observed demineralization. Fermented milk 1 contained a lower amount of total sugars and higher amount of reducing sugars than fermented milk 2, which caused less mineral loss after treatment B. This could indicate that the probiotic, in this experimental model, exhibited some protective effect against enamel demineralization.

**Conclusion**

Under the conditions of this study, the findings demonstrate that fermented milk 2 (Batavito\textsuperscript{®}) can reduce the amount of microorganisms in the oral cavity and mineral loss in bovine enamel. More systematic studies and randomized controlled trials are needed to determine the optimal probiotic strains, daily doses, and vehicles for safely improving oral health.

**References**