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ORIGINAL ARTICLE

Analysis of Chemical Properties of Fermented Milk Beverages Containing Probiotics and Influence on Enamel Demineralization: An *in Vitro* Study

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ABSTRACT

Objective: To evaluate the properties of fermented milk beverages containing probiotics and their potential demineralization on enamel after consumption. **Material and Methods:** Seven fermented milks were analyzed by titratable acidity and pH, and five were considered for erosive cycling. The beverages' calcium (Ca) and Phosphorus (P) were measured. For measurement of potential erosive, bovine dental enamel blocks (n=8) were individually treated (2 min - 4x/day for five days) with 1% citric acid solution pH 3.5 (control) and strawberry, grape, traditional, and orange fermented milk beverages. At treatment intervals, the blocks were immersed in artificial saliva (1h). The samples were evaluated by profilometry and scanning electron microscopy (SEM) at the end of cycling. The results were submitted to ANOVA and 5% Tukey tests. **Results:** For titratable acidity, the traditional flavor had the lowest value, 3.36 ± 0.46mmoles, and the grape flavor had the highest value, 10.7 ± 0.1mmoles. The lowest pH value was the strawberry flavor at 3.61 ± 0.07, and the highest was the orange flavor at 4.10 ± 0.07. The orange flavor showed the highest values of Ca (48.19%) and P (9.69%). The fermented milk beverages promoted surface loss; however, citric acid promoted higher values. **Conclusion:** Fermented milk is an acidic beverage with variations in inorganic components. Their erosive potential is lower compared with citric acid.

Keywords: Tooth Erosion; Cultured Milk Products; Dental Enamel; Probiotics.

Introduction

The search for food quality has increased worldwide, with a consequent increase in the consumption of rich and functional food and beverages, considered sources of essential nutrition that generate health benefits [1,2]. Among these food groups, emphasis is placed on probiotics, which are living microorganisms that can be added to many products (food, medicine, and dietary supplements) and which, when administered in adequate amounts, have beneficial effects on the host's health [3].

The probiotics most commonly used in the food industry are bacteria of the genera *Lactobacillus* and *Bifidobacterium* [4]. Commercial products containing them use supplements and food as vehicles. Regarding the latter, added probiotics are mainly of dairy origin, have significant commercial acceptance, and are marketed in different beverages, such as yogurts and fermented milk [5].

Fermented milk beverages can be defined as a product with or without the addition of other food substances, obtained by coagulating and lowering the milk pH, or reconstituted milk, with or without the addition of different dairy products, by lactic fermentation through the action of specific microorganisms [2]. Microorganisms must be viable, active, and abundant in the final product during its shelf life. The following are considered fermented milk: yogurt, fermented or cultured milk, kefir, kumys, and curd [5]. The benefits for health, in general, are many: synthesis of vitamins, inhibition of pathogens, reconstitution of the intestinal microbiota after the use of antibiotics, increased immunity, the reduced ulcerative activity of *Helicobacter pylori*, non-surgical periodontal therapy, among others [2,3,6].

However, fermented milk beverages have a physicochemical property, low pH [7], typically <4.0 [8], which characterizes it with erosive potential in terms of dental demineralization. Dental erosion is a chemical process of demineralization without the involvement of bacteria, which occurs layer by layer without the formation of a subsurface lesion, causing permanent dental tissue loss, called erosive tooth wear [9,10]. According to the literature, few studies have evaluated the effect of fermented milk on dental demineralization. In an *in situ* study, Lodi et al. [11] verified the drop in biofilm pH and dental enamel demineralization when exposed to fermented milk. Also, some studies have analyzed the effect of fermented milk associated with fluoride therapies or the fluoride concentration in beverages [12] due to the importance of this agent against demineralization.

Another factor that should be highlighted is that fermented milk has large amounts of calcium, which can benefit dental erosion [13,14]. Hara and Zero [15] found that the demineralization capacity of dental enamel by erosion was reduced when calcium was added to beverages. Therefore, properties such as pH, titratable acidity, and inorganic components such as calcium and phosphate ions of probiotics can modulate their erosive potential.

Thus, there are few studies related to the consumption of probiotics and their effects on dental demineralization in terms of dental erosion, even with the increasing use of fermented milk beverages. These previously published studies did not use imaging techniques to verify whether this demineralization increase occurs with the mineral layer's softening and loss. In addition, they did not use an erosive cycling model, no control group with citric acid, and no analysis of the relationship among mineral components.

Considering the potential growth of the fermented milk industry, the different beverages marketed, and the role of these products in the population's health [16], it is essential to analyze the relationship between fermented milk and the different oral conditions that can affect the patients. Information on this topic could provide relevant data to clinicians and researchers, showing whether the daily consumption of these beverages would affect enamel demineralization and the formation of erosive lesions.

Thus, this study aimed to evaluate the properties of fermented milk beverages with the addition of probiotics and the potential effect of demineralizing on enamel in erosive cycling. The null hypothesis tested was that the different fermented milk does not promote enamel surface loss.

Material and Methods

Identification of Fermented Milk Beverages

Fermented milk beverages were identified and purchased from supermarket chains in the city of Maceió, AL, Brazil. Seven beverages were selected. Information about the beverages can be found in Table 1.

Table 1. Beverage information.

Flavor	Commercial Name	Manufacturer
Strawberry*	Chamyto	Dairy Partners Americas Nordeste Produtos Alimentícios LTDA
Grape*	Chamyto	Dairy Partners Americas Nordeste Produtos Alimentícios LTDA
Traditional	Chamyto	Dairy Partners Americas Nordeste Produtos Alimentícios LTDA
Traditional*	Yakut	Yakult S/A Indústria e Comércio
Traditional	Danoninho	Danone LTDA
Orange*	Betânia Kids	Betânia Lácteos S.A.
Orange	Itambé Kids	Itambé Alimentos S.A

*The asterisk indicated the beverages used in the erosive cycling.

Titratable Acidity and pH Analyses

The beverages were selected to include the different options to the public. After that, the beverages were analyzed for titratable acidity and pH. For this, the beverages were opened, and 100 mL (of each product) was inserted in a beaker to analyze the titratable acidity. 0.5 mL of 1 M sodium hydroxide (NaOH) (Dinâmica Química Contemporânea Ltda, Indaiatuba, SP, Brazil) was added to the beverages until pH 7.0. The total volumes of NaOH added were noted to compare mmoles of titratable acidity. This analysis was performed in triplicate.

For pH analyses, a pH electrode calibrated with pH 4.0 and 7.0 buffer standards and connected to a pH meter (Model SA 720, Procyon, São Paulo, SP, Brazil) was used. An aliquot (100 mL) of the fermented milk was placed in a beaker under agitation at room temperature, and its initial pH was determined. The pH measurement was performed in triplicate. After that, four beverages were chosen based on pH value and flavor, and similar beverages were excluded and indicated with asterisks in Table 1.

Analyze the Inorganic Components of Fermented Milk

The beverages chosen were analyzed in relation to inorganic components. Energy-Dispersive X-Ray Spectroscopy (EDX) (7000/8000, Shimadzu do Brasil, Barueri, SP, Brazil) was used for this. An aliquot of each fermented milk was used in this test to obtain the amount of minerals. Each beverage's calcium (Ca) and phosphorus (P) were analyzed.

Bovine Dental Enamel Blocks Preparation

Healthy bovine incisors were collected and stored in 2% formaldehyde, pH 7.0 (Rioquímica S/A Indústria, São José do Rio Preto, SP, Brazil). Crowns were sectioned using double-sided diamond discs (Buehler Ltd, Lake Bluff, IL, USA). Forty dental blocks were prepared, with dimensions of 4 x 4 mm, using an electric cutting machine (Isomet Electric Cutter, Buehler Ltd, Lake Bluff, IL, USA). A digital caliper was used to standardize the dimensions of the blocks. Then, the height of the enamel blocks was reduced by flattening the dentin portion. For flattening and polishing the enamel, sandpaper (3M Brazil, Sumaré, SP, Brazil) was used in descending granulation order (400, 600, and 1200) in a polisher APL-4 (Arotec Ind. Com., Cotia, SP, Brazil) and felt disk (FGM Group, Joinville, SC, Brazil) soaked with diamond solution (Ultradent Products Inc, Alameda Ezequiel Mantoanelli, Indaiatuba, SP, Brazil). Between procedures, dental blocks were washed in distilled and deionized water (Ultrasound, T7, Thornton, Vinhedo, SP, Brazil) for 5 min, and at the end with detergent solution (Buehler Ltd, Lake Bluff, IL, USA), for removal of felt particles and diamond particles. Dental blocks were stored in closed plastic containers, covered with absorbent paper moistened with deionized water, and kept at 100% relative humidity. Blocks with cracks and imperfections were discarded.

Dental Surface Analysis by Profilometry

The baseline analysis of the enamel surfaces was measured using the standards: field of view of 0.25 mm, magnification lens ×20, scan speed ×1, and aperture of 0.4 in XYZ mode (Talysurf CCI MP, Stuttgart, Germany). Subsequently, before exposure to treatments, unplasticized polyvinyl chloride plastic tapes were placed on the sides of dental blocks to protect them from contact with beverages, while the central region (4x1mm) of blocks remained exposed to treatments. This way, comparing the treated area with the untreated one is possible.

Erosive Cycling

For erosive cycling, dental blocks were individually immersed in treatment groups (n=8): fermented milk of strawberry, grape, traditional, and orange. Citric acid 1% with pH 3.5 was used for control. For this, 2.5 mL/mm^2 of exposed dental enamel area was used at 37°C under agitation (100 rpm) 4 times a day [17]. These dental blocks remained immersed in artificial saliva (2.5 mL/mm^2 of exposed dental enamel area), without agitation in intervals between erosive challenges and were again immersed in artificial saliva overnight until the next cycling day. The time of treatments (fermented milk or citric acid - control) was 1 min, and the immersion in artificial saliva was 1 hour, rinsed with deionized water between the phases. The citric acid was prepared with citric acid monohydrate and sodium hydroxide (NaOH) or hydrochloric acid (HCl) (Dinâmica Química Contemporânea LTDA, Indaiatuba, SP, Brazil) for pH adjustment. For the artificial saliva used: 1,5 mM Ca; 0,9 mM PO_4 and KCl 150 mM in buffered solution Tris 20 mM, pH 7,0 (Dinâmica Química Contemporânea LTDA, Indaiatuba, SP, Brazil). Solutions were changed daily. Before the immersion of enamel blocks in treatment solutions and artificial saliva, they were washed with purified water and carefully dried with absorbent paper.

Post Cycling Analysis

The following analyses were carried out on the tooth surface: final profilometry and scanning electron microscopy (SEM) to evaluate the erosive effect of fermented milk on the tooth structure after the 5th day of erosive cycling. For profilometric analyses, the software SPSS Software, version 21 (IBM Corp., Armonk, NY, USA) was used to compare initial and final profiles and obtain the values of loss (μm). For the analyses by SEM, two samples from each group were randomly chosen to be analyzed. The equipment used for image analyses was the VEGA's 4th generation Scanning Electron Microscop (TESCAN do Brasil, São Bernardo do Campo, SP, Brazil) with distances of 250x, 4.00 kx, and 7.00 kx.

Statistical Analysis

For statical analysis, pH and titratable acidity analyses were performed descriptively by calculating the means and standard deviations for prepared samples of each product. Descriptive analysis of results in percentage (% of the Ca and P) was performed for the inorganic components of fermented milk. For the analysis of the effect of fermented milk on the dental enamel structure, the values of loss obtained by profilometric analyses were analyzed about normal distribution by the Shapiro-Wilk test (p>0.05). After that, the data were submitted to analysis of variance (ANOVA) and Tukey's tests, with a 5% significance level.

Results

The titratable acidity (mmoles) and pH of all fermented milk were determined in triplicate, and the results are shown in Table 2. The group that presented the lowest pH was 'Itambé Kids' orange flavor (3.48), while the 'Bethânia Kids' orange flavor group presented the highest pH (4.10). Regarding titratable acidity, the 'Chamyto' traditional flavor group showed the lowest value (3.36), and the 'Chamyto' grape flavor group had the highest value (10.7).

Table 2. Values of titratable acidity and pH of all beverages.

Flavor	Commercial Name	Titratable Acidity	pH
Strawberry	Chamyto	9.98 ± 0.23	3.61 ± 0.077
Grape	Chamyto	10.7 ± 0.1	3.73 ± 0.202
Traditional	Chamyto	3.36 ± 0.46	3.99 ± 0.023
Traditional	Yakut	8.66 ±0.57	3.65 ±0.017
Traditional	Danoninho	5.1 ± 0.1	4.08 ± 0.05
Orange	Betânia Kids	6.16 ± 0.57	4.10 ± 0.072
Orange	Itambé Kids	8.16 ± 0.29	3.48 ± 0.076

The concentrations of minerals found in each fermented milk selected are described in Table 3. The group with the highest Ca and P concentrations was the 'Betânia Kids' orange flavor group (Ca concentrations were 48.197% and P, 9.685%). The groups with the lowest Ca and P concentrations were the 'Chamyto' strawberry flavor, with a Ca concentration of 41.032%, and the 'Chamyto' grape flavor, with a P concentration of 7.326%.

Table 3. Percentage of calcium (Ca) and phosphorous (P) contained in the beverages.

Flavor (Commercial Name)	Ca %	P %
Strawberry (Chamyto)	41.032	8.079
Grape (Chamyto)	41.833	7.326
Traditional (Yakult)	47.195	7.907
Orange (Betânia Kids)	48.197	9.685

The citric acid showed a statistical difference for all groups (p<0.05), demonstrating surface loss (Figure 1) higher than fermented milk. Fermented milk did not differ from each other (p>0.05), with similar surface loss among the different commercial brands, with orange and traditional flavored fermented milk presenting the lowest surface losses among all groups, respectively.

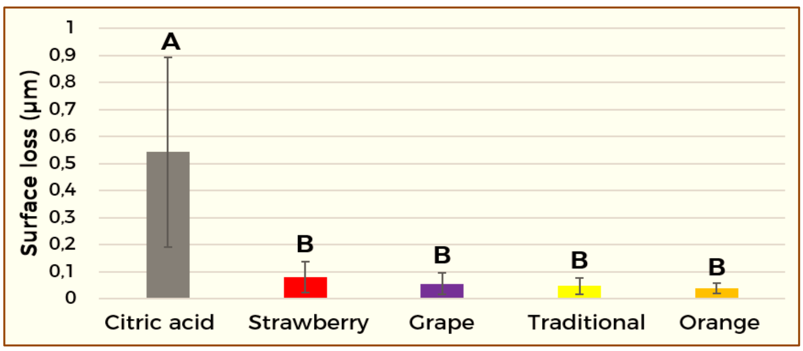

Figure 1. Means of surface loss for all groups tested.

Regarding surface analysis (Figure 2) by SEM, it was observed that the control group (A) had the most significant irregularity in the enamel layer, showing greater consequences from the erosion suffered. Group B had the most eroded surface among fermented milk beverages, showing irregular spikes and valleys. Group C was characterized by an irregular and moderately damaged surface with slight porosity. Groups D and E showed slightly demineralized surfaces, with images that did not show such irregularity in enamel crests.

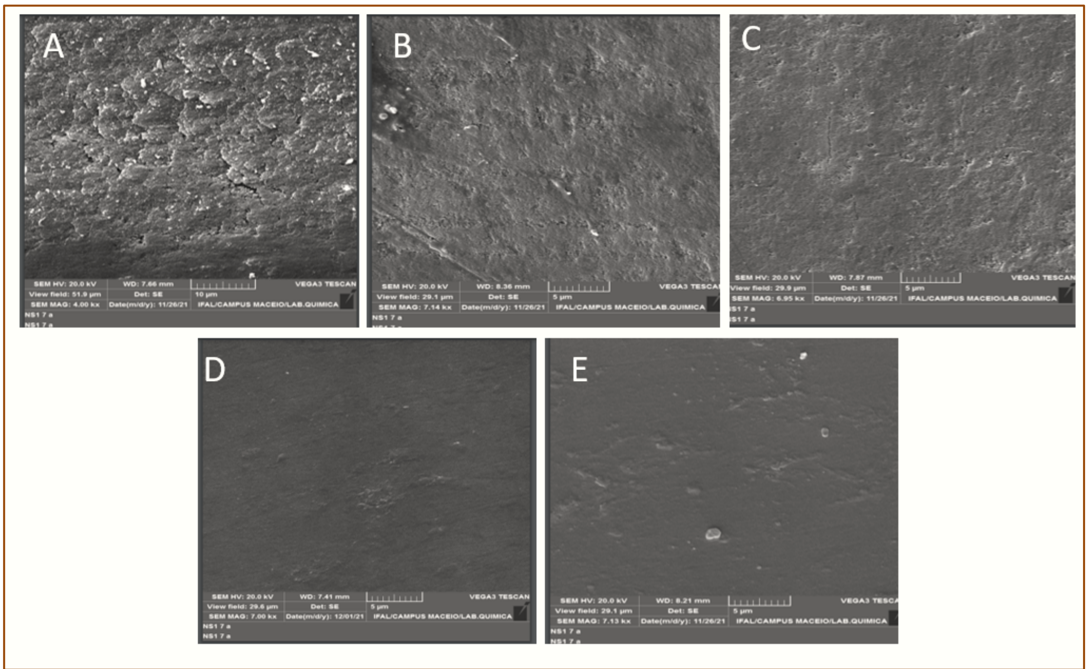

Figure 2. SEM images of the enamel after exposure to different treatments with A citric acid, B strawberry, C grape, D traditional, and E orange.

Discussion

Erosive tooth wear is a condition with higher prevalence nowadays, associated with elevated consumption of acidic beverages [18]. The results of the present study suggest that fermented milk beverages have erosive capacity, with similarity with different flavors. Thus, the null hypothesis was rejected, as there was enamel loss after exposure to the beverages. Through profilometry, a technique that quantifies dental substrate loss [19], dental blocks used in this showed a surface loss. However, compared with citric acid, dental block surface loss was much smaller, showing that citric acid has greater erosive capacity than fermented milk beverages. These results are confirmed in images obtained by SEM. The group that showed the greatest loss in the analysis was the citric acid group, showing significant differences with a decrease above 0.5μm. In SEM images, it was possible to observe the surface with great irregularity.

Despite the pH, the main determinant of dental erosion, all fermented milk beverages containing probiotics showed a pH below the critical value for dental enamel [20], with values ranging from 3.61 to 4.10. Regarding titratable acidity, values ranged from 6.16 to 10.7 mmoles. Beverages with erosive capacity, low pH, and high titratable acidity keep the oral environment acidic for a more extended period and may promote dental demineralization. In addition, other chemical factors are also significant predictors of dental erosion, such as the calcium and phosphorus content in beverages [6].

The analysis of minerals in fermented milk beverages under study showed large amounts of calcium in their compositions, which also greatly influences the erosion process and may explain the difference in erosive capacity in relation to citric acid, which has irrelevant mineral components. Calcium ions are essential to preserve the dental surface. The dissolution of minerals present in the tooth is addressed in the study by Lussi et al. [9], who explain that undersaturated solutions with this substrate cause demineralization; on the other hand, supersaturated solutions do not cause the process of mineral loss of this substance. This occurs by the chemical principle of medium saturation.

All fermented milk beverages had surface loss below 0.1µm, not representing significant differences from each other ($p>0.05$). Although the values of enamel loss did not differ among the flavors, the SEM images showed different patterns of enamel topography. This may be associated with the pH values, mainly in the strawberry group, which is the lowest (pH=3.61). In addition, the inorganic components have influenced their behavior on enamel. Although this group had the second-highest amount of phosphorus in its composition (8.079%), its calcium content was the lowest among the other beverages under study (41.032%). In their research, Barbour and Lussi [21] explained that the amount of calcium has a more significant capacity in the process of reducing the erosive potential of products in their finished commercial form compared to the presence of phosphorus ions. SEM images show irregular surfaces with damage to the enamel morphology.

Despite the grape flavor group, low pH (3.73) and similar calcium (41.833%) and phosphorus (7.326%) contents were observed when compared to the strawberry group. Its titratable acidity was the highest among all fermented milk groups. SEM images showed irregular and moderately damaged surfaces. Thus, its erosive potential is confirmed in the enamel dissolution.

Traditional and orange flavors had the highest calcium (47.195% and 48.197%, respectively) and phosphorus percentages (7.907% and 9.685%, respectively). The orange group had the highest pH among all groups (4.10), although the traditional group had the second lowest pH among groups selected for the final analyses. SEM images concluded that these were the groups that showed the lowest damage to the enamel surface, corroborating values presented, and the profilometric analysis.

The amounts of calcium and phosphorus mineral ions present in fermented milk beverages, as well as their pH and titratable acidity values, directly influenced the erosive capacity of beverages and, consequently, the surface loss of dental blocks under analysis [18]. Larsen and Nyvad [22], in an *in vitro* study, found that enamel dissolution was inversely proportional to the pH of the product under analysis and that even substances with higher pH but with higher titratable acidity also resulted in a high demineralization effect.

These results also corroborate the study by Hara and Zero [15], who analyzed the erosive potential of 10 acidic beverages commercially available, five with calcium supplementation and five without, in which low demineralizing capacity of four beverages containing calcium compared to those that did not was observed, and only one did not show dental erosion reduction. This indicates the influence of calcium ions in reducing a possible demineralization caused by beverages.

However, in the study by Lodi et al. [8] carried out with exposure of dental blocks to two brands of fermented milk beverages with similar amounts of calcium ions in their compositions and to the sucrose control group, it was observed that fermented milk Yakult® (pH=3.51) presented a change in the percentage of surface hardness of blocks similar to sucrose, which has a negligible amount of calcium. In contrast, fermented milk Batavito® (pH=3.87) showed much lower enamel surface demineralization. After its use, fermented milk Batavito® had lower production of insoluble extracellular polysaccharides (IEP) in the dental biofilm compared to fermented milk Yakut®, which could explain its better probiotic action.

The absence of a positive control group, such as non-fermented milk, reduces the chances of extrapolating the results shown in this study. However, considering data from the literature, the pH of non-fermented milk is higher than that of fermented (>6.0) [23], indicating a reduced chance of damage to the enamel surface.

Therefore, the erosive potential of fermented milk can be visualized from an *in vitro* erosive cycling study, which evaluated the surface loss of samples. It should be emphasized that the lowest results of surface loss after exposure to fermented milk beverages can be higher when erosive-abrasive challenges are performed due to the action of mechanical forces on the softened layer [24,25]. In addition, the beverages analyzed in this study can be consumed by children due to the added flavor. It is essential to mention that newly erupted permanent enamel is more vulnerable to acid injury [26]. In association, the high frequency of consumption of the beverages may elevate the values of loss obtained in this study.

On the other hand, it is necessary to highlight the benefits of probiotics for health. Related to the teeth and oral cavity, even with the lower erosive effect on enamel when used in fermented milk, the use of probiotics promoted benefits against pathogens colonization [27]. This is an important topic for caries and periodontal diseases, as shown in previous clinical studies [28,29]. For these advantages, their use is considered a method to improve general and oral health [30,31], nevertheless, with caution in terms of frequency of consumption in the form of milk-based beverages, to minimize the adverse effects on the tooth.

As a strategy to reduce the chances of damage, probiotics can be consumed in different forms in dairy foods. In addition, conscious consumption and attention to patients' dietary habits should be considered preventive measures [32]. These considerations are essential in preventing hard dental tissues since the salivary-acquired pellicle formed by delayed toothbrushing cannot prevent tissue loss [33].

For this, these results can be applied *in vivo* to compare and study the effects of potentially erosive solutions and provide a basis for in-depth studies with living organisms and dietary behaviors, with dental erosion being a multifactorial condition, in which saliva, fluoride, extrinsic factors, and intrinsic factors influence its occurrence.

Conclusion

Fermented milk is an acidic beverage, and characteristics related to its composition, such as Ca and P, may vary according to the flavors. Fermented milk-based beverages generally have a lower erosive potential compared with citric acid.

Authors' Contributions

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All authors declare that they contributed to a critical review of intellectual content and approval of the final version to be published.		

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None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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