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EFFECT OF FERMENTED MILK WITH KEFIR GRAINS ON THE *IN VITRO* DEMINERALIZATION OF BOVINE TOOTH ENAMEL

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Abstract

The dental caries is a progressive destruction of the teeth tissue due to the disbalance in the normal molecule interactions between the enamel and the biofilm, which alters the demineralization-remineralization process. Milk fermentation produces casein-phosphopeptides with proved remineralizing capacity of the enamel. The presence of these peptides in fermented milk with kefir grains has been described. The purpose of this work was to evaluate *in vitro* the capacity of milk kefir to prevent the demineralization of dental enamel.

Bovine incisors (n=68, 17 per group) were treated for 72 h with different solutions: I: artificial saliva at pH 7.2 , II: demineralizing solution at pH 4.5, III: supernatant of kefir fermented milk at pH 4.5, IV: milk supernatant at pH 4.5. The effects of treatments were evaluated by the change in the weight of the specimens, calcium concentration in the solution and by scanning electron microscopy (SEM) of the enamel. Kefir milk supernatant prevented the demineralization process, that was evidenced by a change in weight and calcium concentration that were not different from group I, although the pH was 4.5. In contrast, group IV showed a decrease in weight and an increase in calcium concentration, compared with group I (one way ANOVA, $p<0.05$). Images of SEM agree with the values of weight and calcium concentration. These results indicate that kefir milk supernatant has a protective effect on enamel demineralization *in vitro*.

Key words: kefir, demineralization, tooth enamel.

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Resumen

La caries dental es una patología debido a un desequilibrio en las interacciones moleculares normales entre el esmalte y la biopelícula, que altera el proceso de desmineralización-remineralización. La fermentación de la leche produce fosfopéptidos de caseína con probada capacidad remineralizante del esmalte, y se ha descripto la presencia de estos péptidos en la leche fermentada con granos de kéfir. El propósito de este trabajo fue evaluar in vitro la capacidad del kéfir de leche para prevenir la desmineralización del esmalte dental.

Sesenta y ocho incisivos bovinos (17 por grupo) fueron tratados durante 72 h con diferentes soluciones: I: saliva artificial, pH 7.2, II: solución desmineralizante, pH 4.5, III: sobrenadante de leche fermentada con kefir, pH 4.5, IV: sobrenadante de leche, pH 4.5. El proceso de desmineralización se evaluó mediante el cambio en el peso de las muestras, la concentración de calcio en la solución y microscopía electrónica de barrido (SEM) del esmalte. El sobrenadante de leche fermentada con kéfir impidió el proceso de desmineralización, que se evidenció por un cambio en el peso y la concentración de calcio que no discreparon del grupo I, a pesar de haber tenido un pH de 4.5. En contraste, el grupo IV mostró una disminución en el peso y un aumento en la concentración de calcio, en comparación con el grupo I (ANOVA a un criterio, $p<0.05$). Las imágenes SEM concuerdan con los cambios en el peso y la concentración de calcio en los grupos estudiados. Los datos obtenidos demuestran que el sobrenadante de la leche tratada con kéfir tiene un efecto protector sobre la desmineralización del esmalte in vitro, inducida por el pH ácido.

Palabras clave: *kefir, desmineralización, esmalte dental.*

Introduction

Oral health is an essential part of the overall health and therefore affects the total well-being of people. Dental caries is a disease with high prevalence and public health costs worldwide, despite the use of fluoride and other preventive methods.[1,2] Dental erosion is a multifactorial condition influenced by three main factors: chemical, biological and behavioral. The erosive potential of erosive agents like acidic drinks or foodstuffs depends on chemical factors such as pH, mineral content, clearance on tooth surface, calcium chelation properties, etc.[3]

It is well known that milk and milk products are rich in calcium and phosphorus ions and that they have a high buffering capacity. In addition, milk and its derivatives are a source of biopeptides with beneficial health activity.[4] Fermentation of milk leads to the production of lactic acid and the resulting fall in pH inhibits growth of many pathogenic organisms.[5]

Caseinophospeptides (CPP) are peptidic fractions derived from milk caseins with anticariogenic activity due to the ability to stabilize calcium phosphate on enamel, thus preventing demineralization and promoting remineralization. Most CPP contain a sequence with three phosphoserine residues, followed by two glutamic acids. Negative side chains corresponding to phosphate groups are responsible for binding to minerals, especially calcium.[6]

CPP have become of great interest in the dental field since they can associate to calcium phosphate on the tooth surface to form a pool of calcium and phosphate ions that maintains a state of saturation surrounding the enamel. As a consequence, demineralization is inhibited and the remineralization of enamel is increased.[7] Most of the *in vitro* and *in situ* studies showed strong evidence of CPP bioactivity in the oral cavity.[8]

Kefir milk also known as kefir, originally

from the Caucasus Mountains, is one of the oldest fermented milk. Fermentation is carried out by kefir grains, which contain a varied microbiota composed of lactic bacteria and yeasts and, together with the polysaccharide kefiran,[9] form a symbiotic community that confer unique properties to this beverage.[10]

The consumption of kefir milk has been shown to be as effective as sodium fluoride in the reduction of *Streptococcus mutans* in saliva, supporting the use of modified dairy products for anticariogenic purposes. The presence of phosphopeptides in kefir milk has been recently described and the binding affinity to calcium was confirmed for one of them, but it is estimated that other sequences of the 62 identified of the phosphopeptides could also have mineral binding properties.[11] In addition, kefir milk can be prepared and maintained easily at home, and it constitutes a high calcium and low lactose content food.[12]

To our knowledge, until now there were no studies demonstrating the protective effect of kefir milk on dental demineralization.

The aim of this study was to evaluate the concomitant effect of the kefir supernatant in the presence of acid that arises from milk fermentation.

Materials and methods

For these studies, freshly extracted bovine teeth, which were obtained from animals slaughtered in *CTC* slaughter-house from *Puerto Vilelas* (Province of Chaco, Argentina) were used. Teeth free from caries and enamel defects from bovines that are not older than 3 years old (68 specimens) were included in this study. Crowns were polished with a circular brush with nylon bristles mounted on hand piece and then they were rinsed with distilled water. Teeth were immersed in 5 % formalin for one week at 4°C.

A cross-section at the height of tooth neck was performed to separate the coronal portion from the root with a diamond blade mounted on hand piece with plenty of cooling steady stream of water. The cervical area, the cutting area and the duct of the coronal portion were covered with acid-resistant varnish, leaving only the adamantine tissue exposed. The crowns were stored in saline solution for one week.

This work has been approved by the Ethical Committee of the School of Medicine of Rosario National University.

Treatments

Samples were divided at random in 4 groups and, an adaptation of the method proposed by Ferrazzano to produce enamel erosion was used.[13] The adaptation of the method included: incubation at 4ºC instead of 37ºC and the use of bovine teeth instead of human teeth. The reason for the first modification is that we propose to use samples containing live microorganisms (group III, see below), and at 37ºC the microbial growth and metabolism could not be controlled under our experimental conditions. The second modification is due to the impossibility to obtain the necessary number of human teeth with the adequate quality to carry out the experiment. Each tooth was immersed in 3 ml of solution. Tubes were kept at 4°C for 72 h and at the end of the experiment, measurements were performed on the teeth and the solutions. The following groups were studied:

Group I: samples were immersed in artificial saliva with the following composition: p-hydroxymethylbenzoate (2.00 g/l), sodium carboxymethylcellulose (10.0 g/l), KCl (8.38 mmol/l), $MgCl_2$ (0.29 mmol/l), $CaCl_2$ (1.13 mmol/l), K_2HPO_4 (4.62 mmol/l), KH_2PO_4 (2.40 mmol/l), pH 7.2.

Group II: samples were immersed in demineralizing solution with the following composition: lactic acid (0.1 mol/l), sodium carboxymethylcellulose (0.2 g/l), and pH 4.5. These samples acted as demineralizing control groups.

Group III: samples were treated with supernatant of milk treated with kefir grains,

after centrifugation at 5000 g and 8ºC for 5 min (see below preparation of supernatant from kefir milk). Solutions of Group III had a pH of 4.5 as the consequence of the fermentation process of milk carbohydrate by the kefir grains.

Group IV: samples were immersed in the supernatant obtained from the treatment of milk with lactic acid to obtain a pH of 4.5, and 5 min of centrifugation at 5000 g at 8º C.

Preparation of supernatant from kefir milk

Commercial skimmed milk with 110 mg/100 ml of Ca and desiccated kefir (Prama®, Argentina) was used in the experiments described in this paper. Kefir grains were added at a ratio 5 g per 100 ml of pasteurized milk, 3 % lipid content. The mixture was left fermenting for 24 h at room temperature. The final pH of the solution was measured and if it was higher than 4.5, it was adjusted to such value by adding lactic acid. After milk fermentation for 24 h, it was centrifuged at 5000 g at 8°C for 10 min (Refrigerated centrifugal HERMLE Z 323 K model, Germany). With this procedure, the supernatant was obtained and the precipitate formed by the insoluble fraction was discarded. The supernatant was preserved for the treatment of Group III.

Evaluation Methods

Quantitative Analysis

The effect of solutions on demineralization and remineralization processes was measured through the change in weight and calcium concentration of solutions, after incubation with the crowns. Before and after treatments, the samples were dried until constant weight at 30°C. Each sample was weighted before (t0) and after (tf) the treatment with the solutions. A high precision scale of 0.1 mg error was used for this measurement (Mettler, Switzerland). The difference between weight at (tf) and (t0) in each specimen was calculated. A positive value was interpreted as mineral gain, whereas a negative value was interpreted as predominance of the demineralization process over the remineralization one. Similarly, before (t0) and after (tf) treatment, calcium concentration was measured in the solutions and the difference between tf and t0 was calculated. A positive value of the difference in calcium concentration was interpreted as predominance of the demineralization process over remineralization.

Calcium concentration in the solutions was measured before and after the treatments by atomic absorption spectroscopy with an AROLAB MK II equipment (Metrolab, Buenos Aires, Argentina), using acetylene flame:air in a 1.5:2 ratio. The results were expressed in µg of Ca released or captured during the experiment.

The pH measurements were performed with pH meter Methrom 632.

Qualitative Analysis

Scanning electron microscope (SEM) images were obtained from three specimens of each group. They were placed on platens with vestibular side up and were metalized with a thin layer of gold, by electrical metalization (Sputtering Denton Vacuum Desk II, Moorestown, United States) and were observed by SEM (JEOL JEOL 5800LV. Tokyo, Japan- Electronic Microscopy Service –UNNE). Images were captured digitally with a magnification of x1000 and x3000. (Digitizer Gatan model 788 Digiscan II Pleasanton, United States). Only x3000 images are shown in the result section of this manuscript.

Statistical analyses: statistical analyses were performed using the stat, base and agricolae packages of software R 3.4.4.[14]

Results

Qualitative analysis by SEM indicates that the treatment with kefir milk supernatant has a protective effect against pH induced demineralization. The SEM images of the different treatments are described below.

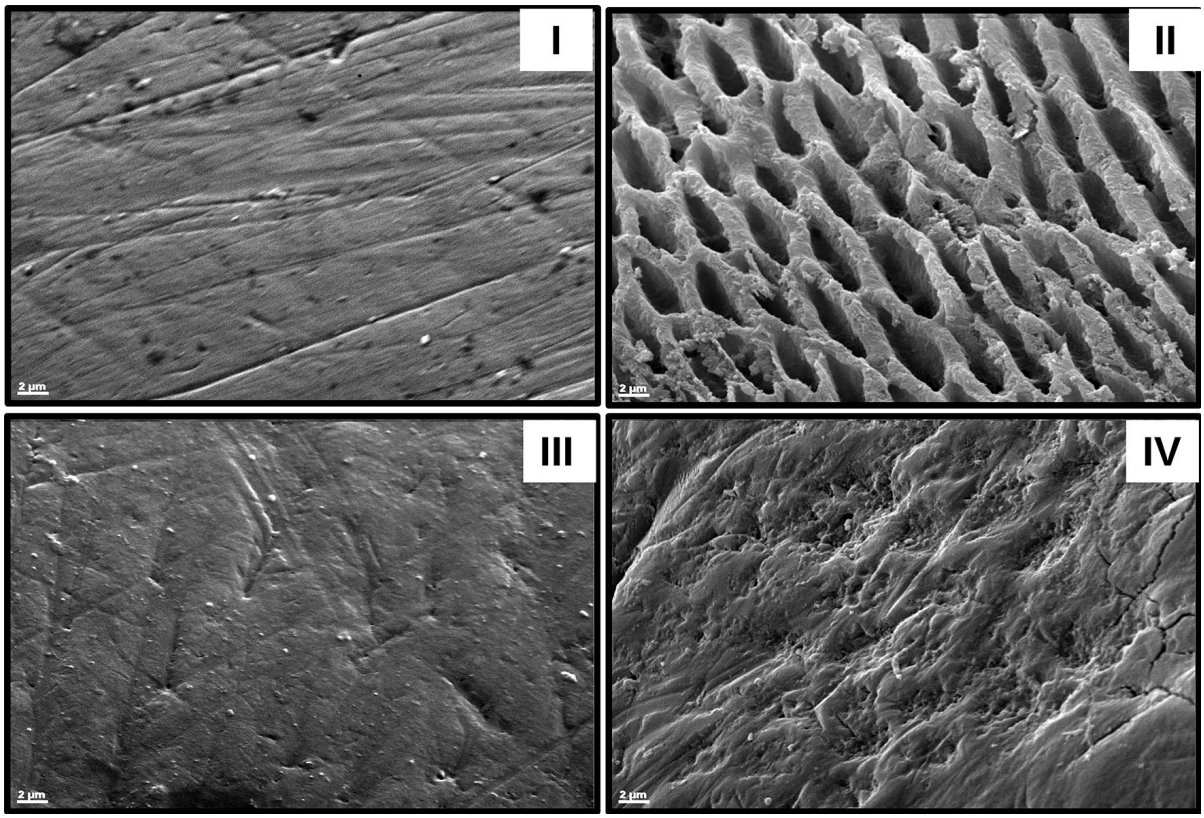

Figure 1: surface of the tooth enamel after 72 h incubation with different solutions. I) healthy enamel of group I, after treatment with artificial saliva, pH 7.2; II) demineralized enamel of group II, after treatment with lactic acid solution at pH 4.5; III) treated with supernatant of kefir milk at pH 4.5; IV) demineralized enamel of group IV: treated with supernatant of milk obtained by treatment of milk with lactic acid, pH 4.5. Images are displayed at 3000x.

SEM evaluation of group I (Fig. 1 I) showed a healthy enamel surface, regular and with uniform texture.

The SEM image of group II (Fig. 1 II) showed the surface micromorphology of artificial lesions created in positive control. The formation of a characteristic honeycomb structure in the demineralized enamel, which exhibits substantial lesions following a specific "as etching" pattern is evident. The core of the prisms was dissolved leaving prominent peripheral margins.

In the SEM images of samples of group III (Fig. 1 III), demineralized areas were not observed. A relatively smooth and even surface, appearing as covered with a mineral deposit is described, possibly this mineral sediment completely filled the previously created lesions. This group showed images of the adamantine surface of relatively smooth appearance and with no undercutting by demineralization.

In the SEM images of group IV (Fig. 1 IV), a heterogeneous surface with large and irregular erosions were observed.

Significant difference was found in weight among treatments applied to bovine tooth enamel (ANOVA, $p < 0.05$). Groups II and IV had a negative weight difference, which implies demineralization of tooth enamel, while groups I

and III had a positive weight difference, meaning that there was no demineralization of tooth enamel. When a post test was performed, a significant difference was found between group I and II, group III differs from group II, and group IV differs from group I. The change in the teeth weight in different groups are consistent with the images obtained by SEM.

Table 1: difference in weight (mg) between the final and initial weight of specimens. At least one equal superscript letter between two columns indicates non-significant differences. * Indicates differences respect Group I, # Indicates differences respect group p<0.05. One way ANOVA, post test LSD.test. Data are shown as mean ± standard deviation.

Group I	Group II	Group III	Group IV
-8.7±5.4	334±534 *	20,3±42,1 #	40,2±65,9 *

A significant difference was found for the difference in calcium among treatments (ANOVA, p <0.05). The difference in calcium concentration in group I, indicates that calcium is not lost by the treatment with artificial saliva. Although groups II, III and IV had a positive calcium difference, group III had the lowest value of calcium lost. This value was not different from group I. On the other hand, groups II and IV differ from group I (LSD test, p <0.05).

Table 2: difference between final an initial calcium concentration (mg/dl). Negative values indicate Ca uptake by the specimen, and a positive values indicate calcium lost from teeth. * Indicates differences respect Group I, # Indicates differences respect group p<0.05. One way ANOVA, post test LSD.test. Data are shown as mean ± standard deviation.

Group I	Group II	Group III	Group IV
5.1±9.9	-2.3±5.1 *	2.8±5.3 #	-0.2±5.8 *

Discussion

The dental caries is a process that implies a lack of balance in the normal molecule interactions between the tooth surface and the adjacent microbial biofilm. If the loss of minerals occurs at a higher speed than the corresponding mineral deposition, it has the potential to lead to enamel cavitation and side effects in dentin and pulp, ending with the localized destruction of tooth hard tissue.[15]

Scientific evidence has shown that the enamel gradual demineralization is normal due to the loss of both calcium and phosphate ions. At the same time, the enamel is remineralized thanks to saliva maintenance, mineral balance, and oral pH. Nevertheless, this balance can be affected, leaning mainly for demineralization.[16]

The enamel remineralization, which occurs physiologically in the oral environment, can be fostered by remineralizing agents or other systems that favor this action. This process has been known for more than a hundred years, but only in recent decades its therapeutic role has been accepted for the control of dental caries.[17]

New enamel remineralizing agents are being considered for the management of patients at high caries risk and the treatment of subclinical lesions and mild white spot lesions, since they provide an alternative to the use of fluoride and the use of dental sealant type materials.[18]

Fermented milk presents a number of beneficial health properties and it is considered a functional food. These beneficial properties can be attributed to the microorganisms that are used in the elaboration of the fermented milk, and to the different products released during the fermentation process.[19] Numerous peptidic fractions with bioactive properties have been isolated from fermented milk.[20]

Biological active peptides are those that exert, additionally to their nutritional aminoacid supply, a physiological effect. These are inactive inside the precursor protein sequence and can be released *in vitro* or *in vivo* by enzymatic hydrolysis.[21]

The data reported in this paper show that supernatant of kefir milk does not have demineralizing effect on enamel *in vitro*. This result is consistent with other studies that indicate a link between dairy consumption and the reduction of dental caries. This effect is mainly attributed to several factors: tooth remineralization, inhibition of bacterial colonization and biofilm inhibition.[22]

Dairy products are the most recognized food group with anti-caries activity.[23] Using human and animal *in vitro* models, the anti-cariogenic activity of milk products was attributed to direct chemical effects of CPP, calcium and phosphate.[24]

The results of this paper indicate that the demineralization of enamel in the presence of the supernatant of fermented milk with kefir grains was significantly reduced *in vitro*. These results were confirmed by SEM micrographs of group I and III where the samples exhibited less surface changes, compared to samples from groups II and IV. Further, our findings are consistent with the proposed mechanism for CPP anti-cariogenic, action, which is associated to CPP interaction with the ACP (amorphous calcium phosphate) on the tooth surface, forming nano-complexes (CPP-ACP) that are incorporated into the dental plaque and stick to the dental surface, acting as calcium and phosphate reservoirs. These nanoparticles of CPP and calcium phosphate, are capable of capturing the excess of free ions and maintain an atmosphere of supersaturation of these ions with respect to enamel, which prevents demineralization and promote remineralization.[25] These peptides have recently been identified in kefir milk, and the calcium binding capability of one of them has been confirmed.[26] Therefore, future research should focus on *in vivo* studies and epidemiological effects of the consumption of functional food in the reduction or elimination of dental caries.

The encouraging results obtained in group III could promote research for the development of preventive measures against dental caries, based on the use of active ingredients derived from food (kefir, yogurt, and cheese) that offer the advantage of being completely biocompatible, easy to obtain, nontoxic, and less expensive than pharmacological treatments.

This work shows that dairy products like kefir milk represent a system capable of preventing demineralization of enamel in its early stages, which strengthen the physiological mechanisms of protection. The identification and characterization of these peptides, being naturally derived from milk, on one hand would allow their use to develop commercial products for oral application

without adverse effects and, on the other hand, would add another functional food feature to kefir, which consumption could be recommended to people suffering from tooth demineralization with therapeutic purposes. Presumably, CPP content in kefir milk is greater than in milk due to proteolytic activity of microorganisms contained in this drink.[27]

Moreover, consumption of probiotic products containing live microorganisms improves oral health. Recently, a pilot study has shown that consumption of kefir is as effective as sodium fluoride in reducing the load of *Streptococcus mutans*. These results support the use of modified milk with anti-cariogenic goal.[28]

It is concluded that kefir milk has a protective effect on enamel demineralization, even at a low pH values. Therefore, although the consumption of kefir is not a method of treatment, it provides a prevention method valid against early enamel demineralization when physiological protection mechanisms are insufficient. It is important to highlight that kefir milk can be prepared at home, as kefir grains are available at food markets.

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