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The performance of probiotic fermented sheep milk and ice cream sheep milk in inhibiting enamel mineral loss

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ABSTRACT

The study aimed to evaluate the effects of two different sheep milk-based food matrices – fermented sheep milk and ice cream – with added probiotic bacteria (*Lactobacillus casei* 431) on dental enamel subjected to an in vitro highly cariogenic challenge. Sixty enamel blocks were selected and randomly allocated into five treatment groups (n = 12): conventional fermented sheep milk (CFSM), probiotic fermented sheep milk (PFSM), conventional sheep milk ice cream (CSMIC), probiotic sheep milk ice cream (PSMIC) and control using deionized water. The blocks were subjected to highly cariogenic pH cycling and the products were applied (5 min), in a blinded way, once a day to simulate a daily use for 8 consecutive days. A microhardness test was performed before and after the treatment to estimate the percentage of microhardness surface loss (% SML). Scanning electronic microscopy (SEM) was performed to confirm the mineral loss. All groups had lost microhardness after the experiment. However, CFSM and PFSM exhibited the most positive findings when compared to the control in both ice creams. Scanning electron microscopy showed less mineral loss in CFSM and PFSM compared with CSMIC, PSMIC and control after the cariogenic challenge. Overall, fermented milk decreased mineral loss from enamel subjected to a highly cariogenic challenge, regardless of the presence of probiotics in their composition, which had a higher efficacy compared to ice cream.

1. Introduction

Probiotics are living microorganisms that are safe for human consumption and, when ingested in sufficient amounts, result in beneficial effects on human health (FAO/WHO, 2012). The most common strains applied in food products belong to the genera *Lactobacillus* and *Bifidobacterium* (Sanders & Marco, 2010). Among lactobacilli family, strains belonging to the species *Lactobacillus casei* are most often used as probiotics (Granato, Branco, Cruz, Faria, & Shah, 2010). Indeed, several beneficial health effects have been attributed to the consumption of L. *casei*, including the inhibition of some pathogens and the improvement of immune responses (Nezhad, Hussain, & Britz, 2015).

Regarding the oral environment, probiotic bacteria have been tested to identify new findings on dental caries prevention and control (Bonifait, Chandad, & Grenier, 2009). Indeed, some new strategies to prevent oral diseases are based on the manipulation of microorganisms, which is provided by probiotics through their inhibitory mechanism on the growth of pathogenic agents (Stamatova & Meurman, 2009), as well as beneficial effects on the cariogenic microflora (Ashwin et al., 2015; Singh, Damle, & Chawla, 2011). However, there is still lack of supported evidence concerning the effects of probiotics on the prevention of enamel demineralization.

Dairy products using cow's milk as raw material are typically the most commonly used food matrices supplemented with probiotic bacteria. These foods are considered effective vehicles for the administration of probiotics, by showing several benefits to the consumer (Lollo, Morato, Moura, Almada, et al., 2015a; Lollo, Morato, Moura, Oliveira, et al., 2015b; Moura et al., 2017). Furthermore, studies have demonstrated that dairy products made from sheep milk are suitable vehicles to deliver probiotic bacteria (Balthazar et al., 2017). Sheep milk has become increasingly popular in the market due to higher yield,

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nutritional value, and higher concentrations of proteins, fats, vitamins and minerals compared with other mammalian milks (Balthazar et al., 2017; Milani & Wendorff, 2011; Park, Juárez, Ramos, & Haenlein, 2007).

The use of probiotic bacteria in sheep milk, the high protein content and milk fat levels allow the production of ice cream with a denser matrix, which results in greater protection for the bacteria against the passage through the gastrointestinal tract, as well as, during the storage of the commercial product regarding the oxygen permeability. The high level of protein is also a favourable feature for the production of fermented milk, as it provides nutrients for microbial growth, leading to a reduction of fermentation time without a direct impact on the manufacturing process (Balthazar et al., 2016). However, studies dealing with probiotic sheep dairy foods are scarce and no information is available related to the impact of these products on oral health. Furthermore, the present study aimed to evaluate the enamel demineralization inhibition potential of two probiotic sheep milk food matrices (fermented milk and ice cream) using a highly cariogenic challenge.

2. Material and methods

2.1. Conventional and probiotic fermented sheep milk and ice cream processing

Whole raw sheep milk containing 5% (v/v) fat (approximately 13% non-fat solids) was obtained from a herd of Lacaune sheep located in the mountainous region of Rio de Janeiro, Brazil. Sheep milk was skimmed to 1.72% (w/w) of fat and finally heat-treated (72–75 °C/15 s) using pasteurizer plates (BCISMINI, EQUILATI, São Paulo, Brazil). The difference in fat levels between the two products is related to subsequent addition of sheep milk cream during ice cream processing, which naturally increased the fat content in the products.

The fermented milk was manufactured following a conventional procedure (Balthazar et al., 2016). Two fermented milks were prepared: conventional fermented sheep milk (CFSM) and probiotic fermented sheep milk (PFSM). To the CFSM was added 1 g/L *Lactococcus lactis* LR-35® (6 log CFU/g, Chr Hansen Valinhos, Brazil), a starter culture without probiotic effects, whereas 1 g/L, according to manufacturer, *Lactobacillus casei* 431 (6 log CFU/g, Chr Hansen, Valinhos, SP, Brazil) a probiotic bacterium, was added to the PFSM. For each treatment, 2 L of semi-skimmed sheep milk (1.72% w/v fat) (Brasil, 2011) was used. Fermentation was carried out at 37 ± 1 °C for 12 h until pH 4.6–4.7 was reached. Subsequently, samples were stored at 5 ± 2 °C in 1-L polypropylene containers.

The ice cream was processed in accordance with standard protocols for conventional and probiotic ice cream (Ferraz et al., 2012). Sheep milk cream (10% fat w/w) was added to skimmed sheep milk (0.33% w/w) together with a mixture of stabilizer and emulsifier (0.99% w/v each) (Selecta®, Two Wheels, Jaragua do Sul, Brazil), sucrose (14.85% w/v) (União, Refined, São Paulo, SP, Brazil) and vanilla essence (0.01% v/v) (Arcolor®, São Paulo, SP, Brazil). The mixture was heated up to 50 °C to dissolve the ingredients and vanilla flavouring (0.01% w/w) was added after the mixture was cooled. Then, ice cream samples were maturated at 5 °C ± 2 for 24 h, and after that, the mixtures were subjected to air incorporation (45%, 3 min, 5 °C, Arpifrio Pro 9, Santo André, SP, Brazil). Finally, the probiotic ice creams were wrapped in propylene containers (v = 200 mL) and stored at − 18 °C.

To the probiotic ice cream was added 1 g/L, according to manufacturer, *Lactobacillus casei* 431 (6 log CFU/g, Chr Hansen Valinhos, Brazil) previously incubated at 37 ± 1 °C for 12 h until pH 4.7–4.6 was reached, whereas the conventional ice cream did not receive any lactic acid and probiotic culture.

2.2. Counts of probiotic and starter cultures

Lactococcus lactis and *Lactobacillus casei* counts were performed in triplicate using Man, Rogosa and Sharpe agar (MRS Agar, HiMedia Laboratories, Mumbai, India) on 1, 14 and 28 days. The proximate analysis was conducted in day 1. Plates were incubated at 37 °C ± 1 °C for 72 h under aerobiosis for L. *lactis* and anaerobiosis for L. *casei* (Souza, Guergoletto, Garcia, & Sivieri, 2011).

2.3. pH measurements

The pH of the products was determined using a digital potentiometer (Model PG1800, Chapter Lab®, SP, Brazil) according to Ferraz et al. (2012). The analyses were performed in triplicate every 7 days for 28 days (n = 4 measurements). Fermented milks remained stored in a domestic refrigerator (5 ± 2 °C), whereas ice creams were stored in a domestic freezer (− 18 °C) for 28 days.

2.4. Proximate analyses

The approximate composition of the products: moisture (%), fat (g/100 g) and proteins (g/100 g) was performed after 24 h after their manufacture using traditional methods (Brasil, 2006). Moisture was determined by drying 5 g of sample at 100–105 °C for 24 h. Fat content was quantified using the Gerber method, and protein level was determined in duplicate by the Kjeldahl method, using a conversion factor of 6.3.

2.5. Calcium values

The calcium content was also determined in duplicate by means of inductively coupled plasma (ICP) optical emission spectrometry (Spectro Analytical Instruments, Kleve, Germany) according to Felicio et al. (2016). Analytical curves were constructed using calcium standards. Ten grams of each sample was acid hydrolysed for approximately 16 h at 120 °C ± 2 °C using 2 mL nitric-perchloric acid solution (2:1 v/v). The samples were then heated in a digestion block (Technal, São Paulo, Brazil) in a fume hood at slow boil to 100 ± 2 °C for 1 h and kept for 2 h at 170 ± 2 °C. After cooling in room temperature, 2 mL of nitric-perchloric acid were added to each tube and heated for 4 h at 170 ± 2C in a digestion block.

2.6. Dental specimen preparation

Initially, 108 enamel blocks (4.0 × 4.0 × 2.0 mm) were obtained from bovine incisors by cutting the buccal surfaces with a diamond disk mounted in a low speed cutting machine (Isomet, Lake Bluff, IL, USA) (Fig. 1A). The blocks were cleaned with distilled and deionized water and embedded in acrylic resin devices. After that, the enamel was sequentially ground using 600 and 1200 grit Al_2O_3 paper in a semi-automatic polisher (model PLF, Fortel, São Paulo, SP, Brazil) (Fig. 1B) in order to obtain a flattened and polished enamel surface in each specimen. After each stage of polishing, samples were cleaned in an ultrasonic machine with distilled and deionized water (Milli-Q®, Merck Millipore Corporation, Darmstadt, Germany) for 5 min (Nassur et al., 2013). Half of the block surface was covered with nail polish to create a control area in order to provide a comparison between the treatment and the control in the same specimen after the experimental protocol (Fig. 1C).

Sample size was calculated in Biostat 5.3 (Instituto Mamirauá, Pará, Brazil) and was based on the mean and standard deviation of a previous study (Lodi, Oliveira, Brighenti, Delbem, & Martinhon, 2015) about the effects of commercial fermented milks on prevention of dental enamel demineralization. Based on a two-sided test, considering a power of 90% and $\alpha = 0.05$, seven enamel blocks in each group were required. With an estimate of 40% loss and the need for two extra samples to be analysed by scanning electron microscopy, where 12 enamel blocks for each group were allocated.

A total of sixty blocks were selected by surface microhardness