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Probiotic Prato cheese consumption attenuates development of renal calculi in animal model of urolithiasis

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ABSTRACT

The effect of probiotic Prato cheese containing *Lactobacillus casei* 01 (7–8 log CFU/g) on urolithiasis was evaluated. Twenty-four male *Rattus novergicus*, Wistar line (8 weeks old), with surgical implant of calcium oxalate tablets (CaOx) were used. The animals were organized into four groups (n = 6) randomly divided into: Naive Control (NC); Control of calcium oxalate (CaOx-C); Calcium oxalate with conventional cheese (CC), Calcium oxalate with probiotic cheese (PC). Urinalysis was performed to evaluate volume, density, pH, urea, creatinine, together with serum urea, creatinine, sodium, potassium and magnesium, and latero-lateral radiographs of the abdominal cavity. Only the PC group presented a significant reduction in the size of the pellets (CaOx-C $\Delta = 1.24$ mg, CC $\Delta = 5.78$ mg and e PC $\Delta = -0.933$ mg). Changes in urinary mineral excretion were observed, with a reduction in potassium (NC = 183.7, Caox-C = 313.8, CC = 108, and e PC = 76.4 mmol/L), calcium (NC = 11.0, Caox-C = 3.8, CC = 4.7 and e PC = 3.4 mg/dL. $p = 0.002$) and magnesium (NC = 11.4, Caox-C = 12.9, CC = 5.7 e PC = 2.4 mg/dL, $p = 0.01$) excretion. Radiological examination confirmed the role of PC in preventing kidney stone development, which support the PC a superior to the current therapeutics, together with a functional ingredient in nutraceutical applications.

1. Introduction

Urolithiasis is a disease prevalent since ancient times dating to around 4.800 BCE (Sebben & Brum, 2007). This pathology promotes high costs to the public health system in several countries deserving a growing attention (Raheem, Khandwala, Sur, Ghani, & Denstedt, 2017). This disease is caused primarily by the formation of calculi (stones) in the urinary system, a higher incidence in kidney (Gottlieb, Long, & Koyfman, 2018). It is suggested that such calculus is formed by the aggregation of polycrystalline compounds wrapped in an organic matrix (Tiselius, 2005) and consist of brushite ($CaHPO_4{\cdot}2H_2O$), hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$), struvite [$(NH_4)Mg(PO_4)(H_2O)_6$] and calcium oxalate [$Ca(COO)_2$], calcium oxalate being the most prevalent (Kaleeswaran et al., 2018).

The mechanisms that lead to the formation of these calculations are complex and still do not have all the routes completely elucidated, mainly due to the heterogeneous composition. However, the development of a correlation between precipitating substances and solubility in the urine, presence of urinary substances promoting the crystallization and concentration of the inhibitors of crystal segregation (Steinberg, 1993).

For urolithiasis patients, diet is a crucial factor in the treatment, since there is a relationship with calcium and oxalate intake and crystal formation (Castro, Reyes, Almaguer, & Valdivia, 2002). Oxalate is a small dicarboxylic acid, which may be derived from the metabolism of various foods, but the oxalate can be found integrally in several plants, often in the form of calcium oxalate crystals (CaOx, De la Huerga López et al., 2005). The increase of calcium intake has a beneficial effect by attenuating the oxalate absorption (Lieske, 2017). In this context, the consumption of cheese is an excellent alternative, since it is an excellent

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source of minerals, mainly calcium (Silva, Balthazar, Esmerino et al., 2018; Silva, Balthazar, Rocha et al., 2018).

According to Hill et al (2014), probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". A great number of potential health benefits associated with the regular intake of probiotic-containing products and the strategies to improve the probiotics efficacy in these foods have been studied (Champagne, da Cruz, & Daga, 2018). Among these benefits, some studies have already shown that ingestion of probiotic is intended to reduce the absorption of food oxalate, as increased oxalate degradation by the intestinal microbiota could create a driving force for the oxalate absorption in the intestine and thus reduce urinary excretion (Lieske, 2017; Sadaf, Raza, & Hassan, 2017; Sönmez, Önal Darilmaz, & Beyatli, in press). In addition, the intake of cheese supplemented with probiotic bacteria has been linked to a variety of health benefits. (Cruz, Buriti, de Souza, Faria, & Saad, 2009), including immunomodulation (Lollo et al., 2012) and improvement in cardiovascular health (Lollo et al., 2015; Sperry et al. 2018).

Prato cheese is a ripened Brazilian cheese, similar to Gouda and Danbo cheeses (Sobral et al., 2016), and corresponds about 20% of all cheese produced in Brazil (Nepomuceno, Junior, & Costa, 2016). This ripened cheese has shown good performance as a probiotic carrier (Silva, Balthazar, Esmerino et al., 2018; Silva, Balthazar, Rocha et al., 2018, 2017). The inclusion of probiotic cheese might have a synergic effect with potential to manage the urolithiasis risk. In this context, the aim of the present work was to evaluate role of probiotic Prato cheese in reducing the urolithiasis risk.

2. Material and methods

2.1. Lactic and probiotic strain

Direct Vat Inoculation (DVI) cultures (lactic starters: *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* R-704; and probiotic bacteria: *Lactobacillus casei*-01) were purchased from (Chr. Hansen, Valinhos, Brazil).

2.2. Cheese processing

The experimental design covered two cheeses: conventional (C) (starter culture only) and the probiotic (PC) (starter and probiotic cultures). Prato Cheese was produced by a traditional manufacturing method as described by Silva, Balthazar, Esmerino et al. (2018), Silva, Balthazar, Rocha et al. (2018), Silva et al. (2017)). The experiment was conducted at the Núcleo Avançado em Tecnologia de Alimentos (NATA), using 120 L of full-fat pasteurized milk (65 °C/30 min). The milk was cooled 32–34 °C and the lactic culture (*Lactococcus lactis* ssp. *Lactis* and *Lactococcus lactis* ssp. *Cremoris* (Sacco, São Paulo, Brazil) was added directly to the milk (1% v/v, 7–8 log CFU/g) and incubated for 40 mins. For probiotic cheese preparation, *Lactobacillus casei*-01 was added together with lactic culture directly to the milk (1% v/v, ~7–8 log CFU/g) and incubated for 40 min. Then, calcium chloride (80 mL/120 L milk), annatto dye (36 mL/120 L milk) and coagulant (Ha La 1175, CHR Hansen Indústria e Comércio, São Paulo, Brazil) were added for milk coagulation within 35–50 min.

The optimal curd set point was determined and the curd was cut into 1 cm cubes and submitted to slow continuous mixing for 15 min, which was followed by removal of part (30%) of the whey and further heating to 42 °C by progressively adding hot water at 80 °C (25 L) to increase the temperature by 1 °C every 3 min. This temperature was maintained until the mass point was reached. Then, curd was placed in rectangular plastic molds (2 kg) and pressed (0.1 MPa for 15 min; 0.24 MPa for 30 min; and 0.31 MPa for 90 min). Cheeses were kept for 5 h at room temperature. The cheese samples were then dried at 12 °C for 72 h, and vacuum-packed and stored at 12 °C for 25 days for ripening.

2.3. Probiotic and lactic acid bacteria counts

Twenty-five grams of conventional and probiotic cheese samples were mixed with 225 mL sterile 0.1% peptone water (w/v) (Oxoid Brasil LTDA, São Paulo, Brazil), homogenized, and subjected to 10-fold serial dilutions. The microbial counts were carried out in duplicate using the pour plate technique. The culture medium M17 agar (Oxoid Brasil LTDA, São Paulo, Brazil) was used to enumerate *Lactococcus lactis*, incubated aerobically at 37 °C/72 h. The enumeration of *L. casei*-01 was performed in duplicate under anaerobic conditions using MRS agar (Oxoid Brasil LTDA, São Paulo, Brazil) containing vancomycin at 37 °C/72 h (Silva, Balthazar, Esmerino et al., 2018; Silva, Balthazar, Rocha et al., 2018).

2.4. Proximate composition, calcium and sodium levels

The proximate composition (moisture, protein, and fat; g/100 g) and the mineral contents (Ca and Na) was determined using conventional methods (Silva, Balthazar, Esmerino et al., 2018; Silva, Balthazar, Rocha et al., 2018). Moisture was determined by oven-drying 5 g sample at 100–105 °C for 24 h. Protein was determined by the Kjeldahl method, and fat was quantified by the Gerber method. For the determination of minerals, the cheese samples (1.5 g) were placed in 500 mL Kjeldahl flasks, and concentrated HNO_3 and $HClO_4$ (3:1 v/v) was added and left to stand for 30 min. The samples were mineralized (Buchi K-439, Flawil, Switzerland) until a colorless solution was obtained. Finally, the material was cooled and transferred to a volumetric flask containing 50 mL ultra-pure deionized water (Millipore, Darmstadt, Germany). Ca and Na levels were determined by atomic absorption spectrometry in an air-acetylene flame using the iCE 3000 Series Atomic Absorption Spectrometer (Thermo-Scientific, Hemel Hempstead, Hertfordshire, UK), and a deuterium lamp (SMI-Labhut Ltd., Churcham, Gloucester, UK) for background correction and cathode lamps (SMI-Labhut Ltd.) suitable for each element.

2.5. Clinical trial

2.5.1. Animals

For *in vivo* experimental model twenty-four male *Rattus novergicus*, Wistar lineage (8 weeks old) were employed. This study was approved by the Animal Experimentation Committee from Federal University of Grande Dourados (UFGD) (31/2015). The animals furnished by UFGD's laboratory animal center were kept under controlled conditions (22 °C temperature; 40–60% humidity and a cycle dark-light of 12 h). They were individually identified and was kept in polypropylene cages with food and water ad libitum.

The experimental design was developed to analyze the effects of twenty-five days probiotic Prato cheese intervention on *in vivo* urolithiasis model. The animals were randomly by initial weight and assigned to 1 of 4 groups (n = 6 per group): Naïve control (NC); Calcium oxalate control (CaOx-C); Calcium oxalate with conventional cheese (CC); Calcium oxalate with probiotic cheese (PC). The animals in cheeses groups consumed 2 g/day/animal of respective cheeses during 25 days (Fig. 1). The NC and CaOx-C groups received only water and chow. Euthanasia was practiced one day after the metabolic cage to collect 24-h urine samples.

2.5.2. Preparation of calcium oxalate pills

Calcium oxalate (CaOx) pills were obtained through supersaturation, adding 23.34 g calcium chloride and 19.92 g potassium oxalate mixed by constant grip to 300 mL distilled water during 2 h under agitation at 75 °C. Mixture was kept under agitation at 75 °C and rinsed 10 times for removing the excess of potassium chloride. The resulting mixture was placed in a greenhouse at 37 °C for 2 weeks for the crystal growing and then transferred to molds (4 mm diameter per 1 mm height) in order to obtain the CaOx crystal pills. They were