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Action of lactoferrin on the multiplication of *Lactobacillus casei in vitro* and in Minas fresh cheese

Ação da lactoferrina na multiplicação de *Lactobacillus casei in vitro* e em queijo Minas frescal

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Abstract

This study evaluated the activity of lactoferrin on the multiplication of probiotic *Lactobacillus casei in vitro* and in Minas fresh cheese. Growth curves of *L. casei* in BHI broth containing 1 mg/mL and 2 mg/mL of lactoferrin were performed. Additionally, Minas fresh cheeses added of *L. casei* (control) and added of *L. casei* and lactoferrin (2 mg/g and 4 mg/g) were produced and stored at 5°C during 28 days. Cheeses were analyzed for pH, titratable acidity and enumeration of *L. casei* and psychotropic microorganisms in days 1, 7, 14, 21 and 28 and for the centesimal composition in day 1. The experiment was repeated three times, and data were analyzed by ANOVA and Tukey test at 5% significance level. When tested *in vitro*, *L. casei* multiplication was stimulated by lactoferrin at a concentration of 2 mg/mL, but this activity has not been verified in the cheese, even in that added by lactoferrin at 4 mg/g. Psychotropic population in the cheeses added by lactoferrin did not differ from control cheese ($P>0.05$), demonstrating that no antimicrobial activity occurred in the products. More studies should be performed to evaluate the antimicrobial effects of lactoferrin in foods, since they have more variables that may affect the activity of this protein when comparing to *in vitro* tests.
Key words: Antimicrobial activity, biologic activity, cheese, milk products, probiotics

Resumo

O presente estudo avaliou a atividade da lactoferrina sobre a multiplicação de *Lactobacillus casei* probiótico *in vitro* e no queijo Minas frescal. As curvas de crescimento de *L. casei* em caldo BHI contendo lactoferrina nas concentrações de 1 mg/mL and 2 mg/mL foram construídas. Adicionalmente, queijos Minas frescal contendo somente *L. casei* (controle) e *L. casei* e a lactoferrina (2 mg/g e 4 mg/g) foram produzidos e armazenados a 5°C durante 28 dias. Foram realizadas as análises de pH, acidez livre titulável e enumeração de *L. casei* e microrganismos psicrotróficos no dia 1 (dia da produção) e após 7, 14, 21 e 28 dias de armazenamento. Além disso, no dia 1, foram realizadas as análises de composição centesimal. Os experimentos foram realizados em triplicata e os dados obtidos foram analisados utilizando-se ANOVA e teste de Tukey a 5% de significância. Nos ensaios *in vitro* a multiplicação de *L.*

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casei foi estimulada pela presença da lactoferrina na concentração de 2 mg/mL. Entretanto, esta atividade não foi observada no queijo, mesmo quando a concentração de 4 mg/g de lactoferrina foi utilizada. A população de microrganismos psicrotróficos presentes nos queijos adicionados de lactoferrina não diferiram das populações observadas para os queijos controle (P>0,05), demostrando que não houve atividade antimicrobiana nos produtos. Estudos futuros devem ser conduzidos para avaliar os efeitos antimicrobianos da lactoferrina em alimentos, pois são muitas as variáveis que afetam a atividade desta proteína quando comparados aos resultados obtidos *in vitro*.
Palavras-chave: Atividade antimicrobiana, atividade biológica, queijo, probióticos, produtos lácteos

Introduction

Lactoferrin (LF) is an iron-binding glycoprotein and member of the transferrin family, mainly found in milk (LEVAY; VILJOEN, 1995). It is a protein with a molecular mass of approximately 80 kDa, formed by two lobe counterparts linked by an alpha helix, with binding sites for ferric iron (JENSSEN; HANCOCK, 2009). This protein has a wide variety of biological functions normally associated with the body's defense (SHIMAZAKI, 2000), such as immunomodulatory, antitumor, antioxidant and antimicrobial effects (WARD; URIBE-LUNA; CONNEELY, 2002; JENSSEN; HANCOCK, 2009).

The antimicrobial activity was the first function of LF to be discovered (REITER; ORAM, 1967; ARNOLD; COLE; MCEGHEE, 1977; REITER, 1978). For a long time, it was believed that the only mechanism of antimicrobial activity of lactoferrin was due to its ability to sequester iron, an essential nutrient for microorganisms (ALMSTÅHL; WIKSTRÖM; GROENINK, 2001; SGARBIERI, 2004). However, research has shown that there are highly cationic areas on the surface of LF, which exert potent bactericidal effect by interacting with elements of the bacterial membrane negatively charged, such as lipopolysaccharides of Gram negative bacteria and probably teichoic and lipoteichoic acids of Gram positive bacteria (APPELMELK et al., 1994; GONZALEZ-CHAVEZ; AREVALO-GALLEGOS; RASCON-CRUZ, 2009).

Lactoferrin not only inhibits the proliferation of pathogenic and spoilage microorganisms, but it also promotes population growth of other organisms, such as some lactic acid bacteria (MITSUOKA, 1990). According to Coppa et al. (2006), lactoferrin from breast milk supports the predominance of bacteria which require low concentrations of iron for growth, such as *Lactobacillus* and *Bifidobacterium* of the child intestinal microflora. Although this mechanism of action is not yet been elucidated (COPPA et al., 2006), many studies suggest that the growth stimulatory activity of LF may be related to the presence of lactoferrin-binding proteins on the surface of the bacterial membrane (KIM et al., 2004).

Thus, LF may be a mechanism for iron acquisition if the bacteria have external membrane receptors capable of binding to the LF-iron complex specifically, which results in the internalization of the metal (MODUN; MORRISSEY; WILLIANS, 2000; KIM et al., 2004). Studies conducted by Bezkorovainy and Topouzian (1981) show that the iron required by the bacteria is strain-dependent and the lactoferrin-induced proliferation is heterogeneous among different strains.

In the last decade, dairy products containing bovine LF and probiotic microorganisms have been developed (KENJIN, 2002; RAHMAN et al., 2010). The addition of LF to promote the population growth of probiotic *Lactobacillus* in dairy products is an attractive option for consumers, who could count on the benefits provided by LF and also by the probiotic microorganism. So, the purpose of this study was to evaluate if lactoferrin can promote the proliferation of probiotic *Lactobacillus casei* either in the culture medium and in Minas fresh cheese matrix, that is a product manufactured without preservatives added, low salt concentration and, consequently,

with a short shelf life. The antimicrobial activity of lactoferrin on the microbiological spoilage of the cheese was also evaluated.

Materials and methods

Preparation of inoculums

In order to carry out *in vitro* test, the strain of probiotic *Lactobacillus casei* (Danisco, Dangé, France) was reactivated in BHI broth (Brain Heart Infusion, Himedia, Mumbai, India) and incubated under aerobic conditions at 37°C for 48 h. After incubation it was seeded on MRS agar (De Man, Rogosa and Sharpe, Himedia) and the plates were incubated under the same conditions. The culture of *L. casei* added to each cheese was reactivated in milk the day before the manufacture. For this purpose, skimmed milk powder (Molico, Nestlè, Araçatuba, Brazil) was reconstituted in water at 80°C (1:10), cooled to 37°C and added of 2% of the lyophilized culture. The mixture was incubated at 37°C for 3 h and then stored at 5°C for 18 h.

Growth curve of Lactobacillus casei in culture medium

Three test tubes containing 5 mL of BHI broth were prepared as following: 1) control broth (C); 2) addition of lactoferrin (Sigma-Aldrich, St. Louis, USA) at a concentration of 1 mg / mL (LF1) and 3) addition of lactoferrin at a concentration of 2 mg / mL (LF2). Each one of the tubes was added by approximately five small colonies (<1mm) of *Lactobacillus casei* taken from the MRS agar plates prepared early.

After homogenization, an aliquot from each tube was collected and the absorbance was read in a spectrophotometer (Biomat 3 Thermo Scientific, San Jose, USA) at 660 nm. Then, the tubes were incubated at 37°C for 48 h and the absorbance was read every 120 minutes. The entire experiment was repeated five times.

The enumeration of *L. casei* was performed in all the tubes at the beginning (0 h) and at the end of the curve (32 h) by homogenizing 0.1 mL broth with 0.9 mL sterilized saline solution (0.85%) (Synth, Diadema, Brazil). From this initial dilution, a number of decimal dilutions were prepared using the same diluent. A 1 mL aliquot was collected from each dilution and plated in depth in MRS agar. After 48 h at 37°C under aerobic conditions, the colonies were counted and the results were expressed in CFU/g (FRANK; CHRISTEN; BULLERMAN, 2005).

Minas fresh cheese manufacture

Pasteurized whole milk (Mamele, Arapongas, Brazil) was standardized with pasteurized skim milk (Frimesa, Marechal Cândido Rondon, Brazil) to obtain a final fat content of 3.0%. The standardized milk was heat treated at 65°C for 30 min, cooled to 37°C and added of *L casei* culture, which was prepared according to cited previously. After 30 min of incubation at 37°C, 50% calcium chloride solution (0.25 mL / L, Synth Diadema, Brazil) was added to the milk, followed by the addition of 0.25 mL / L lactic acid (85% food-grade solution, Kinetics Reagents and Solutions, Jandira, Brazil), and rennet powder (Bela Vista, Alto da Bela Vista, Brazil) sufficient to coagulate the milk within 30 min, as described by Wolfschoon-Pombo (1980). After 30 minutes, the curd was cut. After cutting, the curd remained without agitation for five minutes and then submitted to slow mixing for 3 min, followed by a removal of part of the whey (30%). Sodium chloride (15 g / L, Cisne, Cabo Frio, Brazil) was added, followed by a settling period of 2 minutes. Another partial whey removal was conducted by removing approximately one third of the initial total whey. The curd with the remaining whey was weighed and divided into three equal parts. Lactoferrin was added to two of the parts, in proportions of 2 mg/g (LFC2) and 4 mg/g (LFC4) and the third part was considered the control sample

(CC). The cheeses were placed in plastic molds under refrigeration (5°C) and turned every 15 min (three turns) and then stored at 5°C for 12 h. The cheeses were then fractionated, sealed under vacuum in plastic bags and stored at 5° C for 28 days until the time of analysis. The entire experiment was repeated three times.

Physicochemical composition of milk and cheeses

The milk employed on Minas fresh cheese manufacture was analyzed for fat content, pH and titratable acidity. The cheeses were evaluated for protein, lipids, ash, carbohydrates, total solids and salt content on day 1 and for pH and titratable acidity on days 1, 7, 14, 21 and 28. The analyses were performed according to Association of Official Agricultural Chemists methods (AOAC, 2003). The pH was measured using an immersion electrode (Tecnal, Piracicaba, Brazil) previously calibrated. The titratable acidity was determined by titration with a solution of 0.1 N sodium hydroxide (Kinetics Reagents and Solutions, Jandira, Brazil) using phenolphthalein as indicator. The nitrogen content was determined by the Kjeldahl method and the nitrogen values were multiplied by the factor 6.38 to obtain the equivalent amount of protein. The ash content was measured by incineration at 550°C in a muffle furnace (FDG Equipment, EDGCON 1P, São Paulo, Brazil) and total solids were determined by gravimetric method at 105°C for 16 h using a forced air circulation drying oven (Nova Ética, Vargem Grande Paulista, Brazil). The fat content was determined by Gerber method (AOAC, 2003) and the salt content by Volhard method. The lactose content was calculated by subtracting the values of lipid, protein and ash from the total solids content.

Microbiological analyses of Minas fresh cheese

The enumeration of *Lactobacillus casei* was performed after 1, 7, 14, 21 and 28 days after cheese manufacture. For this purpose, portions of 10 g of each cheese sample was homogenized in appropriate plastic bags (Nasco Whirl-Pak, Fort Atkinson, USA) with 90 mL of sterilized saline solution (0.85%) (Synth, Diadema, Brazil). From this initial dilution, a number of decimal dilutions were prepared using the same diluent. A 1 mL aliquot was collected from each dilution and seeded in depth in MRS agar. After 48 h at 37°C under aerobic conditions, the colonies were counted and the results were expressed in CFU / g (FRANK; CHRISTEN; BULLERMAN, 2005).

For the enumeration of psychrotrophic bacteria, 0.1 mL aliquots were seeded on agar surface for plate counting (Himedia). The plates were incubated at 7°C and the number of colonies was counted after 10 days and multiplied by 10 and by the inverse dilution factor of the respective plate. The results were expressed in CFU/g (SWANSON et al., 1992).

Statistical analysis

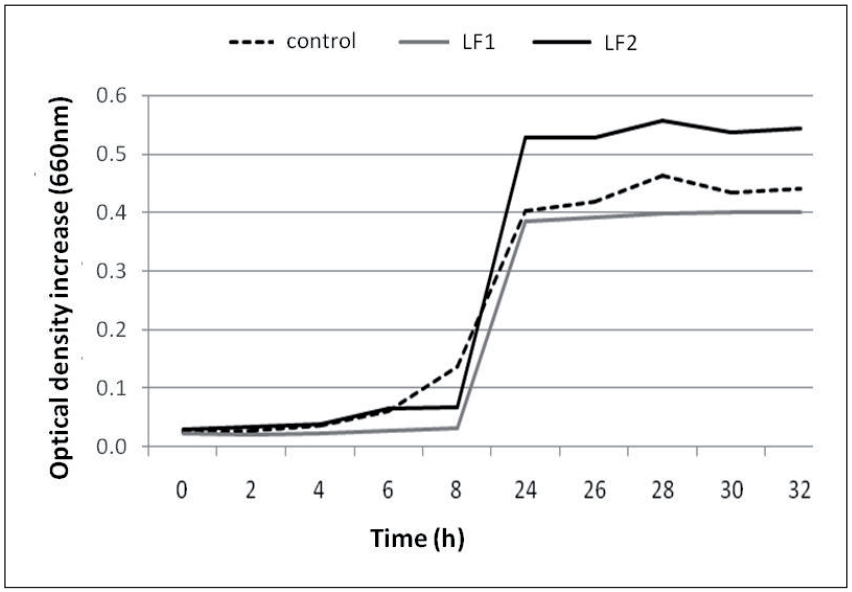
The results were evaluated by analysis of variance (ANOVA) and Tukey test at 5% significance level using Statistica software (STATSOFT, 2000).

Results and Discussion

Growth curves for Lactobacillus casei in culture medium

By observing the optical density values of BHI broth incubated at 37°C either control broth (C – without lactoferrin supplementation) and added lactoferrin broths (LF1 and LF2) presented an increase in *L. casei* populations ($P<0.05$) after 32 h (Figure 1). No difference was observed when the control sample was compared to LF1 ($P>0.05$). The broth containing 2 mg/mL of lactoferrin (LF2) presented a growth promotion in *L. casei* population ($P<0.05$), confirmed also by the initial (0 h) and final (32 h) enumeration for the probiotic microorganism. The increase observed in populations of *L. casei* after 32 h at 37°C were 5.32, 5.33 and 6.48 log CFU / mL in C, LF1 and LF2, respectively.

Figure 1. Increasing of optical density (660 nm) due to the increased population of *Lactobacillus casei* in BHI broth (C – control broth) and in BHI broth supplemented with 1 mg lactoferrin / mL (LF1) and 2 mg lactoferrin / mL (LF2) for 32 h at 37°C.



Source: Elaboration of the authors**.**

The results indicated that the stimulatory activity of lactoferrin on *L. casei* appears to be dose dependent, since this probiotic microorganism was stimulated only in the broth containing the highest concentration of protein in its native form (2 mg/mL). Different results were reported by Kim et al. (2004), who evaluated the influence of apo (iron free), holo (iron saturated) and native form of lactoferrin in the multiplication of *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis* and *Bifidobacterium bifidum*. These authors observed a growth promotion activity of LF for all strains, which was more modest for *Bifidobacterium* than *L. acidophilus*. Furthermore, the degree of iron saturation did not influence the *Bifidobacterium* species but influenced *L. acidophilus*, whose growth was stimulated by holo-LF (1 mg / mL) and native LF (0.5 mg/mL) but not by apo-LF.

On the other hand, Tian et al. (2010) studied the action of LF in concentrations of 1.25, 2.5, 5, 10, 20 and 40 mg/mL on the multiplication of the probiotic microorganisms *Lactobacillus acidophilus, L. plantarum, L. reuteri, L. rhamnosus, Bifidobacterium lactis* and *Pediococcus acidilactici in vitro*. The researchers found no inhibitory or stimulatory action of lactoferrin at the doses tested.

Physicochemical properties of Minas fresh cheese

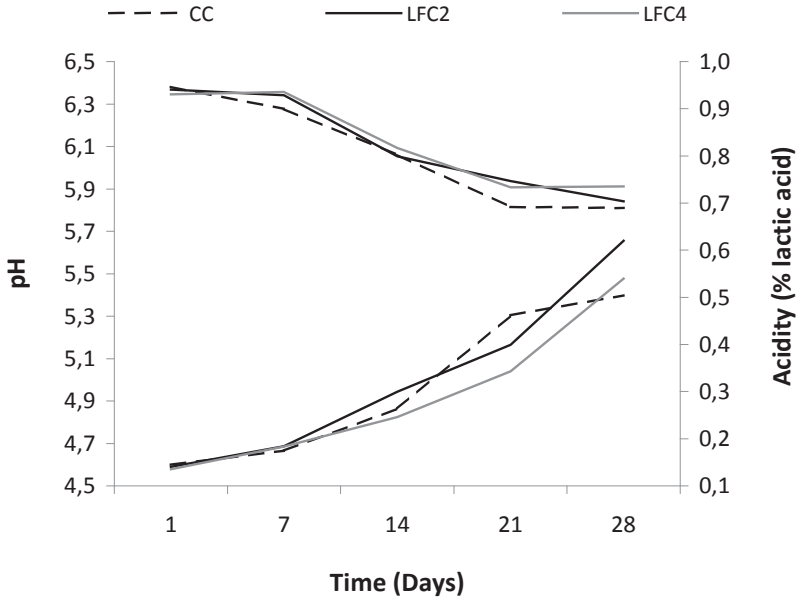
Despite the wide variation in the composition of the Minas fresh cheese, the protein and lipid values observed in this study are within the range mentioned by several authors: 11-15% and 12-21% respectively (ALEGRO, 2003; BURITI; ROCHA; SAAD, 2005; SOUZA; SAAD, 2009). The salt content was lower than the values observed by other authors (1.4 to 1.6%) (DORNELLAS, 1997; CAMPOS, 2000).

When Minas fresh cheeses CC, LFC2 and LFC4 were compared, no significant differences ($P>0.05$) were observed in the composition. The mean composition of cheeses were 65.80% moisture, 2.67% ash, 1.25% salt, 14.25% protein, 13.17% fat and 4.11% carbohydrates. According to these results, the cheeses were in accordance with

the official standards required by the Technical Regulation of Identity and Quality of Minas fresh cheese (BRASIL, 2004), which establishes that it must be a cheese with very high moisture (above 55%).

Moreover, the pH values decreased and the titratable acidity increased for all the cheeses during 28 days of refrigerated storage (P<0.05) (Figure 2). This behavior was due to the multiplication of psychrotrophic and probiotic microorganisms, which degraded the lactose with consequent acid production. When cheeses LFC2, LFC4 and control were compared, no difference was detected concerning these parameters (P>0.05).

Figure 2. Mean values of pH and titratable acidity of control probiotic cheese (CC), supplemented with 2 mg/g lactoferrin (LFC2), and supplemented with 4 mg/g lactoferrin (LFC4) during 28 days of refrigerated storage (5°C).

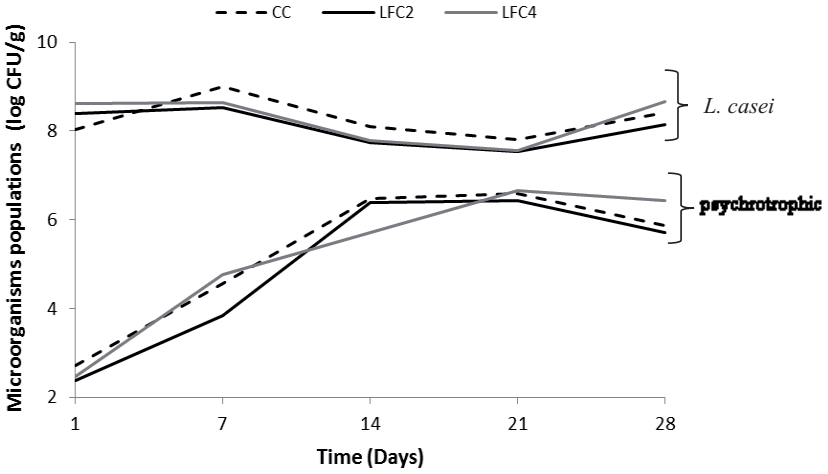


Source: Elaboration of the authors**.**

The pH values of cheeses ranged from 6.38 to 5.81 during 28 days of storage. Although most bacteria multiply best at pH near 7, *Lactobacillus* can tolerate lower pH levels. Buriti et al. (2005) found an increase in the population of *L. paracasei* in Minas fresh cheese acidified with lactic acid and stored at 5°C for 21 days, with pH values ranging from 6.57 to 5.99.

Concerning *L. casei* populations, no significant differences were detected (P > 0.05) among cheeses CC, LFC2 and LFC4. The average population of *L. casei* during refrigerated storage of cheeses at 4°C, ranged from 7.54 and 8.99 log CFU/g to LFC2 and CC, respectively (Figure 3). These results show there was no positive or negative influence of lactoferrin on the growth of *L. casei* during 28 days in the conditions tested (P>0.05).

Figure 3. Mean populations of *Lactobacillus casei* and psychrotrophic microorganisms of control cheese (CC), supplemented with 2 mg/g lactoferrin (LFC2), and supplemented with 4 mg/g lactoferrin (LFC4) during 28 days of refrigerated storage (5 °C).



Source: Elaboration of the authors**.**

Although the multiplication of probiotic bacteria did not occur during the storage of cheeses, populations of *L. casei* remained above the minimum viable concentration suggested by researchers to confer positive effects on the health of consumers, which are typically 10^6 CFU/g of a product (SHAH, 2007).

Several authors have evaluated the multiplication of probiotic microorganisms in the culture medium promoted by LF (GRIFFITHS et al., 2003, KIM et al., 2004; RAHMAN et al., 2010; TIAN et al., 2010). Studies on the effect of lactoferrin in cheeses have been carried out aiming to evaluate the antimicrobial activity of this protein (KENJIN, 2002; QUINTIERI et al., 2012), but its ability to promote the multiplication of probiotics has been little studied.

Since Minas fresh cheese is not a matured product and presents soft and raw mass, high moisture, low salt content and low acidity, it allows the development of many microorganisms, such as spoilage, which use the nutrients from food and produces lactic acid and other by-products. Pathogenic bacteria such as *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus* may also grow and cause infection or intoxication from food sources (FOX et al., 2000).

Thus, one of the main factors that limit the shelf life of Minas fresh cheese is the microbial growth (LOGUERCIO; ALEIXO, 2001). The storage temperature (4 °C or 5 °C) of this type of cheese inhibits the multiplication of microorganisms except psychrotrophic bacteria, which can grow at refrigeration temperatures regardless of its optimum growth temperature (COUSIN, 1982).

The psychrotrophic are an important group of microorganisms which develop in refrigerated food products for periods of one to four weeks (PERRY, 2004) and can cause product deterioration. The main psychrotrophic bacteria that spoil milk products belong to the genus *Pseudomonas* (FRANK; CHRISTEN; BULLERMAN, 2005), which produce extracelular proteases and lipases at refrigeration temperatures (ARCURI et al., 2008; TEBALDI et al., 2008). These enzymes are responsible for proteolysis, lipolysis and changes in taste, aroma and color of the cheeses (PERRY, 2004; CANTONI; BERSANI, 2010).

Figure 3 shows that the population of psychrotrophic microorganisms increased approximately 4 log cycles between the day 1 and 28 of storage ($P<0.05$), with average initial and final counts of 2.5 and 6.6 log CFU/g, respectively. This result indicates that lactoferrin was not effective in controlling the deteriorating microbiota of Minas fresh cheese.

Quintieri et al. (2012) evaluated the effect of bovine lactoferrin hydrolyzated by pepsin (LFH) on the natural microbiota of three commercial mozzarella cheeses (A, B and C) stored at 4 °C for seven days. The authors observed that the inhibitory activity of LFH depends not only on the bacterial population studied but on the sample analyzed. Sample A showed no difference in the populations of mesophilic microorganisms and *Pseudomonas* of the control cheeses and cheeses added by LFH at concentrations of 0.4 mg/mL and 2 mg/mL. However, it was found their antimicrobial activity in relation to the coliforms, once the highest concentration tested showed effect in a shorter period of time. In sample B, at least at some time, all microorganisms were inhibited by LFH. Only coliforms were not inhibited in the sample C.

The antimicrobial activity of LF in cottage cheese artificially contaminated with *Escherichia coli* was evaluated by Kenjin (2002). The author compared the populations of *E. coli* in three cheeses: control cheese, cheese added by native LF and cheese added by glycosylated LF. It was verified a population growth in all the cheeses, which were stored at 15 °C for nine days. From the sixth day there was a significant difference between treatments ($P<0.05$). The increase in the population of *E. coli* was approximately 1.5, 2 and 1 log cycles in the control cheese, cheese added by native LF and cheese added by glycosylated LF, respectively. The results showed that the molecule of native LF was not effective in controlling population of *E. coli* in the cheeses.

In this study, the addition of lactoferrin (2 mg/mL) was able to stimulate the growth of *Lactobacillus casei* in the culture medium but not in the cheeses, even when added twice the effective concentration in BHI broth. One hypothesis for this behavior is the presence of psychrotrophic microorganisms that produce enzymes with proteolytic activity which may have degraded the lactoferrin added (MOUSHUMI; SOMKUTI, 2010). Regarding psychrotrophic bacteria, the bactericidal effect of lactoferrin may have been affected by pH, by the presence of cations and/or by the population of these microorganisms (DEL OLMO; MORALES; NUÑEZ, 2008).

When tested *in vitro*, lactoferrin at a concentration of 2 mg/mL stimulated the growth of *L. casei*, but the same effect was not observed in the cheeses added of 4 mg/mL lactoferrin. Also, psychotropic population in the cheeses added by lactoferrin did not differ from control cheese, demonstrating that no antimicrobial activity occurred in the products. More studies are needed, since foods have other variables that may affect the action of lactoferrin.

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