Proteolysis in goat “coalho” cheese supplemented with probiotic lactic acid bacteria

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ABSTRACT

This study aimed to analyse the proteolytic effects of adding isolated and combined probiotic strains to goat “coalho” cheese. The cheeses were: QS – with culture Start, composed by Lactococcus lactis subsp. lactis and L. lactis subsp. cremoris (R704); QLA – with Lactobacillus acidophilus (LA-5); QLP – with Lactobacillus paracasei subsp. paracasei (L. casei 01); QB – with Bifidobacterium animalis subsp. lactis (BB12); and QC, co-culture with the three probiotic microorganisms. The cheeses were analysed during 28 days of storage at 10 °C. The probiotic cell count was higher than 6.5 and 7 log colony-forming units (CFU) g⁻¹ of cheese at the 1st and 28th days of storage, respectively. The addition of co-culture influenced (p < 0.01) proteolysis in the cheese and resulted in a higher content of soluble protein and release of amino acids at the 1st day after processing. However, over all 28 days, the cheese supplemented with Bifidobacterium lactis in its isolated form showed the highest proteolytic activity, particularly in the hydrolysis of the alpha-s2 and kappa-casein fractions.

1. Introduction

“Coalho” cheese is a highly appreciated product because of its sensory properties, including a slightly salty and acid flavour, mild aroma, and compact and soft texture. This cheese has been produced and consumed for over 150 years and has great importance in the economy of goat milk-producing regions, especially for small producers with no access to industrial facilities for milk processing (Oliveira, Garcia, Queiroga, & Souza, 2012; Queiroga et al., 2013; Silva et al., 2012).

Goat milk presents some specificities related to its chemical properties, specially due to the characteristics of its proteins, which display reduced levels, or even a lack of, alpha-casein, as well as structural differences in alpha-lactalbumin and beta (β) lactalbumin. These characteristics make it less allergenic when compared to bovine milk. Therefore, cheeses prepared using goat milk present a number of desirable properties to many consumers, especially those who are allergic to the type of protein present in bovine milk (Albenzo & Santillo, 2011).

Researchers have dedicated special attention to goat “coalho” cheese because it is considered a functional food, especially due to its peptide profile and antioxidant activity (Silva et al., 2012). Furthermore, cheeses such as “coalho” cheese, are products with peculiar characteristics which protect probiotic bacteria against oxygen, and also against low pH and bile salts, when going through the gastrointestinal tract. This group of characteristics, which also includes, amongst others, a pH close to neutral, a normally high level of water activity (which clearly depends on the amount of salt in the cheese and on the maturation conditions, in case the product is matured), a solid matrix (which facilitates the “insertion” of bacteria) and a relatively high fat concentration make these products more adequate as probiotic vehicles when compared to fermented milk and yoghurt (Bergamini, Hynes, Quiberoni, Sauárez, & Zalazar, 2005). In literature, some studies have already demonstrated the potential of “coalho” cheese as a carrier matrix for probiotic lactic bacteria, enabling the count of microorganisms such as Lactobacillus acidophilus, Lactobacillus paracasei subsp. paracasei and Bifidobacterium animalis subsp. lactis at the end of shelf life, in accordance with recommendations for this kind of product (at least 10⁷ CFU) (Garcia, Oliveira, Queiroga, Machado, & Souza, 2012; Madureira et al., 2005; Oliveira et al., 2012; Santos et al., 2012). This quantity (10⁷ CFU) is the minimum number of bacteria...
required at the moment of ingestion, in order to ensure a favourable impact on consumer health (De Vuyst, 2000; Talwalkar, Miller, Kailasapathy, & Nguyen, 2004).

The probiotic genera most frequently used in different traditional cheeses from Brazil, including “Minas” cheese, “Coalho” cheese, and cream cheese, are *Bifidobacterium* and *Lactobacillus* (Burns et al., 2012; Gomes et al., 2011; Rodrigues et al., 2011). The addition of the strains *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. animalis* subsp. *lactis* in coalho” cheese, which already presents inherent advantages regarding the composition of its proteins, is an option in order to refine the final quality of the product, enhancing its technological, physicochemical and sensory profiles and thus making it more attractive to consumers (Escobar et al., 2012). In addition to these benefits to the nutritional matrix, probiotics also have beneficial effects on consumer health, when ingested in the correct amount. Amongst these benefits are an improvement in the immune system (Lollo et al., 2012) and the strengthening of intestinal immunity (Modzelewskaa-Kapitula, Kobukowski, & Klebukowski, 2010).

The nutritional and sensory improvements in probiotic cheeses are related to the wide spectrum of enzymes that are contained in probiotics, catalyse biochemical reactions over the period of cheese storage, and lead to the production and release of different compounds that affect the quality of the final product, especially the texture and flavour (Albenzio et al., 2013; Randazzo, Pitino, Ribera, & Caggia, 2010).

Such biochemical reactions include a set of protein-related events known as proteolysis. The proteolytic process involves the action of enzymes naturally found in the milk, coagulant agent and microbial enzymes produced by lactic acid bacteria intentionally added during cheese-making. Proteolysis involves the destabilisation of the casein micelle through the release of peptides and amino acids that undergo a catabolic process, thus forming other volatile compounds such as amines, acids (isobutyric, isovaleric and valeric), thiols, esters and others (Garcia et al., 2012; Steele, Broadbent, & Kok, 2013; Wolf, Perotti, Bernal, & Zalazar, 2010).

The potential of goat “coalho” cheese as a functional food, especially as a food matrix source of different probiotic bacteria (such as *L. acidophilus*, *L. paracasei*, and *Bifidobacterium lactis*), has been reported in literature (Oliveira et al., 2012; Santos et al., 2012; Silva et al., 2012).

However, extensive studies on the proteolytic changes caused by the activity of probiotic bacteria added during the processing and storage of goat “coalho” cheese (probiotic and conventional) have not been described. Thus, this study aimed to investigate the effects of proteolytic activity resulting from the addition of *L. acidophilus* (LA-5), *L. paracasei* subsp. *paracasei* (*L. casei* 01) and *B. animalis* subsp. *lactis* (BB 12), in isolated and combined form, to goat “coalho” cheese. The bacteria with probiotic effects were selected according to the optimal viability in the matrix according to literature (Oliveira et al., 2012; Santos et al., 2012).

2. Materials and methods

2.1. Cultures and reagents

Five goat “coalho” cheese formulations were processed in different batches in triplicate using lyophilised commercial cultures...
Fig. 2. Protein fractions of goat “coalho” cheese supplemented with probiotic lactic acid bacteria and starter culture over 28 days of storage at 10 °C ± 2 °C. QS – with starter culture; QLA – with Lactobacillus acidophilus; QLP – with Lactobacillus paracasei; QB – with Bifidobacterium lactis; QC – with co-culture.

Fig. 3. Reversed-phase HPLC of goat “coalho” cheeses supplemented with probiotic lactic acid bacteria and starter culture over 28 days of storage at 10 °C ± 2 °C. QS – with starter culture; QLA – with Lactobacillus acidophilus; QLP – with Lactobacillus paracasei; QB – with Bifidobacterium lactis; QC – with co-culture.
(Chr. Hansen, Valinhos, São Paulo, Brazil). The cultures were added (100 mg of each culture per 1 L of milk) to the different formulations: QS (Lactococcus lactis subsp. lactis and L. lactis subsp. cremoris – R704, batch 3128520); QLA (L. acidophilus – LA-5, batch 3139352); QLP (L. paracasei subsp. paracasei – L. casei-01, batch 3089189); QB (B. animalis subsp. lactis – BB 12, batch 3100870); and QC (L. acidophilus, L. paracasei subsp. paracasei and B. animalis subsp. lactis at a ratio of 1:1:1). The starter culture was only added to the QS formula.

The reagents and chemical products used for the analyses were obtained from laboratory suppliers (Sigma–Aldrich Chemie GmbH, Steinheim, Germany).

### 2.2. Cheese-making protocol

For the preparation of each cheese treatment, 10 L of refrigerated and pasteurised (65 °C for 30 min) goat milk obtained from native breeds that belong to the Cooperative of Farmers of Monteiro, Paraíba (“Cooperativa de Produtores Rurais de Monteiro” – CAPRIBOM) were used. Initially, the milk was heated to 90 °C for 10 min, then cooled to 45 ± 1 °C and then treated by direct acidification with lactic acid (0.85 mL 100 mL−1) at 0.25 mL L−1. The lactic acid bacteria cultures were added in the concentration of approximately 250 g capacity), which were maintained at 36 °C under 10 °C ± 2 °C. QS – with starter culture; QLA – with Lactobacillus acidophilus; QLP – with Lactobacillus paracasei; QB – with Bifidobacterium lactis; QC – with co-culture.

Initially, 25 g of cheese was added and homogenised in 225 mL of peptone water (1 g L−1) and subjected to serial dilutions (10−1 to 10−5). Then, 0.1 mL aliquots of each dilution of the samples were transferred to plates containing DeMan-Rogosa-Sharpe (MRS) agar supplemented with 5% cysteine and incubated at 37 °C for 48 h under anaerobiosis (Anaerobic System Anaerogen, Oxoid Inc., Ogdensburg, NY, EUA). The count was expressed as the number of colony-forming units per gram of cheese (log CFU g−1) (FDA, 1992).

### 2.4. Analysis of soluble protein and nitrogen fractions

The cheeses were analysed for their insoluble protein, total nitrogen, non-protein nitrogen and non-casein nitrogen contents. The insoluble protein concentration was determined by the Folin method using bovine serum albumin as the standard (Lowry, Rosebrough, Farr, & Randall, 1951). The total nitrogen (TN) (991.20) and trichloroacetic acid (TCA)–soluble nitrogen (SN) contents (991.21) were determined using the Kjeldahl method following the methodology by Association of Official Analytical Chemists (2010). The soluble nitrogen (SN) content at pH 4.6 was determined according to Andreatta et al. (2007). Proteolysis was evaluated according to the extent of the proteolysis index (EPI) and depth of the proteolysis index (DPI) using the following
equations: 
\[ EPI = \frac{(SN \text{ at pH } 4.6)}{TN} \times 100, \]
\[ DPI = \frac{(TCASN)}{TN} \times 100 \] (Andreatta et al., 2007).

2.5. Electrophoretic profile analysis

The electrophoretic profile analysis was performed using the technique described by Laemmli (1970). The stacking gel was prepared at a concentration of 3.5% polyacrylamide in 0.5 M Tris–HCl buffer, pH 6.8, and 1% sodium dodecyl sulphate (SDS), whereas the separation gel was prepared by forming a gradient of 7.5–17.5% polyacrylamide in 3 M Tris–HCl buffer, pH 8.8, and 1% SDS. The samples of protein extracts were prepared according to the methodology described by Egito et al. (2002). The electrophoretic run was performed under constant amperage (25 mA), and at the end of the run, the gel was removed from the plate and fixed in 12.5% TCA for 1 h and then stained with Coomassie brilliant blue R – 250 at 0.005%. The excess dye was removed with a destaining solution of methanol, acetic acid and water (1:3.5:8 v/v/v). The molecular weights of the protein fractions in the cheese samples were compared using a 12–225 kDa molecular weight marker (GE Healthcare Life Sciences, Piscataway, NJ, USA).

2.6. Peptide hydrophobicity analysis

The separation of peptides by hydrophobicity was performed using a Nova-Pak C18 column (4.6 m × 250 mm, 4 µm particle size, cartridge; Waters, Ireland) connected to a high-performance liquid chromatography system (Varian, Waters 2690, California, EUA). The injection volume of the soluble extract (0.2 g/mL) was 20 µL, and the mobile phase was composed of eluent A (ultrapure water with 1% trifluoroacetic acid) and eluent B (acetonitrile with 1% trifluoroacetic acid). A linear gradient of eluent A and eluent B was applied for 60 min with a flow rate of 1 mL min⁻¹, and detection was performed at 218 nm.

2.7. Profile of free amino acids

The free amino acids of the evaluated cheeses were extracted by orbital shaking for 60 min with 0.1 M chloric acid (g mL⁻¹) followed by pre-column phenyl isothiocyanate (PITC) derivatisation according to White, Hart, and Fry (1986) and Hagen, Frost, and Augustin (1989). The separation of phenylthiocarbamyl amino acid (PTC-aa) derivatives was performed in an HPLC system (Shimadzu Corporation, Tokyo, Japan) and Luna C18 reversed-phase column (250 mm × 4.6 mm, 5 µm; Phenomenex Inc., Torrence, CA, USA). The mobile phases consisted of an acetate buffer at pH 6.4 and a 40% acetonitrile solution. The sample was injected automatically (50 µL), and detection was performed at 254 nm. The chromatographic separation was performed at a constant flow rate of 1 mL min⁻¹ at a temperature of 35 °C. The chromatographic run time was 45 min, the results were expressed in mg of amino acid per 100 g of “coalho” cheese, and the quantification was performed by adding the α-aminobutyric acid internal standard.

2.8. Statistical analysis

The statistical analysis of the data was performed using an analysis of variance (ANOVA) followed by factorial analysis up to the
significance level of 1–5%. The Assistat software, beta version 7.6 statistical package was used (Silva & Azevedo, 2009). A principal component analysis (PCA) was performed using Unscrambler® X.1 (CAMO S.A.) and coded in Matlab 6.5 (Mathworks, USA).

3. Results and discussion

3.1. Viability of lactic acid bacteria

Differences were not observed (p > 0.05) between the lactic acid bacteria counts in the control goat “coalho” cheese and probiotic goat “coalho” cheese over the 28 days of storage. The lactic acid bacteria counts in the cheeses were higher than 6.5 log CFU g⁻¹ cheese and 7 log CFU g⁻¹ cheese at the 1st and 28th days of storage, respectively. In addition, the five cheeses showed lactic acid bacteria counts higher than 7 log CFU g⁻¹ after 7 days of storage. The minimum probiotic bacteria count to ensure a positive impact on health is 7 log CFU g⁻¹ (De Vuyst, 2000; Talwalkar et al., 2004).

3.2. Soluble protein and proteolysis analysis

The cheeses produced with the probiotic cultures and starter culture showed an increase (p < 0.01) in the soluble protein content over the 28 days of storage (Fig. 1A). Compared with the other cheeses, the “coalho” cheeses processed by adding a starter culture and B. lactis showed higher concentrations (p < 0.01) of soluble proteins over the 28 days of storage.

The increased soluble protein content in the cheese is directly related to the EPI and release of peptides and amino acids. Red smear cheeses reported in literature, such as Xinotyri Greek goat cheese (Bontinis, Mallatou, Pappa, Massouras, & Alichanidis, 2012), Reggiano Argentinone cheese (Wolf et al., 2010), Gokceada goat cheese (Hayaloglu, Tolu, & Yasar, 2013), and Urfa sheep’s milk cheese (Kirmaci, Hayaloglu, Ozer, Atasoy, & Turkoglu, 2014), show a behaviour similar to that obtained in the present study.

Among the analysed probiotic lactic acid bacteria, B. lactis exhibited the most intense proteolytic activity and released the highest amount of amino acids and hydrophilic peptides during storage, thus increasing the concentration of soluble proteins (Albenzio et al., 2013).

The four probiotic “coalho” cheeses presented maximum proteolysis indices by the 7th day of storage, and the value remained constant until the 28th day. However, the control sample showed a different behaviour, and cheese prepared with the starter culture exhibited a maximum proteolysis peak on the 14th day after processing, which then decreased until day 21, with subsequent stabilisation of the proteolytic process until the end of the storage period (Fig. 1B).

The enzymes present in the coagulant agents added during cheese production had a greater effect on the EPI; nevertheless, in addition to “coalho”, enzymes of microbial origin affected the initial proteolysis. Among the microbial enzymes, those of probiotic origin presented higher proteolytic activity compared with the enzymes originating from the starter culture (Rodrigues et al., 2011).

The DPI of the analysed cheeses differed (p < 0.01) between the cultures and analysed times (Fig. 1C). The cheese prepared with a co-culture of probiotics had a higher index for all evaluated times, and this trend may have been related to the synergistic effect of the probiotic cultures on the proteolytic potential. However, the cheese processed with the starter culture exhibited a lower DPI compared with the probiotic cheeses. Thus, the probiotic strains produced greater proteolytic activity than conventional strains used for cheese-making (Albenzio et al., 2013).

3.3. Electrophoretic profile analysis

Fig. 2 presents the protein fractions in the five goat “coalho” cheeses analysed over the 28 days of storage. The most abundant casein fractions detected in the cheeses were αs2 and β-casein, which release peptides of high, medium and low molecular weight as well as amino acids when hydrolysed (Oliveira et al., 2012; Randazzo et al., 2010). In goat cheese, β-casein is predominant, and this fraction is important for forming a firmer coalho over a shorter period of time (Steele et al., 2013).

The αs2-casein is found in the milk of mammals and at high concentrations in goats (Selvaggi, Laudadio, Dario, & Tufarelli, 2014). In turn, αs1-casein may or may not be detected in goat cheese, which is related to the genetics of the goats (Ollalía et al., 2009). The low level of this fraction is desirable because it will result in a less allergenic milk. The proportion of αs1-casein in goat and bovine milks is the major difference in their protein profile. During cheese processing and storage, peptides are released through the proteolytic activity of enzymes. However, the characteristics of released peptides are a result of the milk protein composition and enzymes involved in this reaction. The released peptides may exhibit biological activities, such as antioxidant and antimicrobial activities (Costa et al., 2014).

The proportion of αs2-casein detected in the evaluated cheeses exhibited reduced concentrations during storage, thus indicating that these cheeses can be consumed by individuals with milk protein allergies (Albenzio & Santillo, 2011). The alpha (αs2) and kappa (κ) casein fractions were the most hydrolysed during storage compared with the other fractions detected in bands for goat “coalho” cheese.

The protein fraction κ-casein is important because it is located in the outer region of the casein micelles, which are responsible for stabilising the structure (Selvaggi et al., 2014). Since the 1st day after processing, κ-casein showed a steep decrease in the cheeses produced with B. lactis and co-culture; however, in the other cheeses, especially the cheese supplemented with L. acidophilus, the degradation of this protein fraction was observed by the 7th day. This early decrease most likely indicates the intense proteolytic activity of B. lactis and co-culture as a result of its presence.

The decreased intensity of the αs2-casein fraction band indicated the higher proteolytic activity of B. lactis among the evaluated lactic acid bacteria. Albenzio et al. (2013) analysed the electrophoretic profile of Scamorza sheep’s milk cheese and found a high degree of α-casein hydrolysis by B. lactis compared with cheese prepared with the conventional culture.

Because of the resistance to degradation in β-casein, differences were not detected in the degree of hydrolysis of this fraction between the processed cheeses. This resistance is considered desirable from a sensory perspective because the hydrolysates resulting from the casein fraction have a bitter flavour (Kirmaci et al., 2014). α-Casein is more easily degraded compared with β-casein, and this result has also been detected in other studies with cheese (Bontinis et al., 2012; Hayaloglu et al., 2013).

3.4. Peptide hydrophobicity analysis

The activity of proteolytic enzymes results in the release and degradation of protein-chain peptides. The hydrophobic behaviour of peptides extracted from the five analysed cheeses is presented in Fig. 3. The separation of peptides was performed using a linear gradient (100–0%) of water for 60 min; thus, the peptides eluted from 0 to 30 min were hydrophilic and from 30 to 60 min were hydrophobic. Hence, the goat “coalho” cheeses have a hydrophilic peptide profile.

The total area of the chromatographs of the peptide profile (except for that of the cheese supplemented with the co-culture)
showed an increase (p < 0.01) over the 28 days of storage (Fig. 4): 90% for the control cheese, 194% for the cheese processed with *L. acidophilus*, 52% for the cheese processed with *L. paracasei* and 41% for the cheese processed with *B. lactis*.

Figs. 3 and 4 indicate that the cheese prepared with *L. acidophilus* presented a higher (p < 0.01) proportion of released peptides at the end of storage compared with the other cheeses. The higher peptide production by *L. acidophilus* most likely resulted from the larger production of endopeptidases by the probiotic micro-organisms and increased activity of these enzymes (Albenzio et al., 2013).

Compared with the goat “coalho” cheese in the QLA treatment, the cheeses prepared with *L. paracasei*, *B. lactis* and the co-culture showed an increase (p < 0.01) until the 14th day of study and decrease at the 28th day. This decrease is most likely the result of the more intense proteolysis of these cheeses caused by the activity of lactic acid bacteria used in their preparation. The lactic acid bacteria used in this study (*L. paracasei*, *B. lactis* and co-culture) are known to produce exopeptidases that act in the N- and C-terminal regions of the peptide chains and release a single amino acid residue (Hayaloglu et al., 2013).

3.5. Analysis of the free amino acid profile

The addition of probiotic lactic acid bacteria and the storage time influenced the release of amino acids in the goat “coalho” cheeses (Fig. 5). On the first day after processing, the co-culture promoted high proteolytic activity, thus increasing the concentration of free amino acids. However, this release was increased over time by the activity of probiotic bacteria of the genus *Lactobacillus*. Michaelidou, Katsiari, Voutsinas, Kondyli, and Alichanidis (2003) found that bacteria of this genus have high proteolytic activity as a result of peptidase action.

Among the 18 quantified amino acids, 7 essential amino acids (isoleucine, leucine, phenylalanine, lysine, tryptophan, valine and tyrosine) and 2 non-essential amino acids (proline and arginine) stand out in the free amino acids profile of the goat “coalho” cheeses. The production of free amino acids contributes to the characteristic flavour of cheeses and acts as precursors for other catabolic reactions that produce keto acids, ammonia, amines, aldehydes, acids and alcohols, which are essential contributors to the cheese taste and aroma (Iličić et al., 2012). Phenylalanine and tyrosine are two of the main amino acids that participate in the Streecker degradation reaction, which also leads to the formation of aromatic compounds that affect cheese aroma (Irigoyen, Ortigosa, Juan Saras, Oneca, & Torre, 2007).

At 28 days of storage, the arginine concentration was highest when the co-culture was used, which is an important finding because this amino acid acts as a vasodilator when converted to nitric oxide; thus, it is involved in the control of high blood pressure (Potje et al., 2014). A PCA was applied to evaluate the behaviour of the probiotic lactic acid bacteria according to the release of free amino acids throughout the storage of the goat “coalho” cheese (Fig. 6). The principal components PC1 and PC2 explained 61% and 15% of the total variance, respectively.

The analysis of Fig. 6 indicates that all the cheeses (with the exception of the cheese produced with the co-culture) showed correlations on the 1st day of storage. Because of its synergistic effect, the co-culture produced a high release of amino acids by the 1st day of storage, and this effect is particularly apparent in the presence of the amino acids arginine, aspartic acid, glutamic acid and glycine, which are located on the positive axes of PC1 and PC2. The levels of these amino acids decreased during QC cheese storage, which was most likely because of the use of these free amino acids as a substrate for the co-culture.

The amino acids released by the proteolytic enzymes may undergo a conversion process (deamination, decarboxylation and dehydrogenation) and then catabolism by the bacterial cultures (Sinz & Schwab, 2012).

The evaluation of the positive axis of PC1 indicates that the cheese prepared with *L. acidophilus* produced the greatest changes in the release of free amino acids over the entire storage period compared with the other evaluated cheeses. The cheeses that exhibited the highest inter-correlations were QS with QB and QLB with QC; however, these cheeses exhibited lower free amino acid production.

4. Conclusion

Goat “coalho” cheese prepared with different probiotic cultures presented advantages in the proteolytic process relative to cheese prepared with the starter culture (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremaster*). The combined use of probiotic cultures promoted increases in the soluble protein content and presented a greater release of amino acids by the 1st day after processing. The probiotic goat “coalho” cheeses exhibited a maximum EPI after 7 days of storage, and its αs2 and κ-casein fractions displayed a higher degree of hydrolysis during storage. Among the cheeses prepared with isolated bacteria, *B. lactis* exhibited stronger proteolytic activity, higher soluble protein content and higher degradation of the αs2 casein fraction during storage. Future studies should focus on the action of these probiotics on the lipolysis and flavour/aroma formation of probiotic goat “coalho” cheeses.

Conflicts of interest

The authors declare no conflict of interest.

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