Food Chemistry 196 (2016) 359–366

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Proteolysis in goat "coalho" cheese supplemented with probiotic lactic acid bacteria

Taliana Kênia Alves Bezerra [a], Ana Rita Ribeiro de Araujo [a], Edilza Santos do Nascimento [a], José Eduardo de Matos Paz [a], Carlos Alberto Gadelha [a], Tatiane Santi Gadelha [a], Maria Teresa Bertoldo Pacheco [b], Rita de Cássia Ramos do Egypto Queiroga [a], Maria Elieidy Gomes de Oliveira [a], Marta Suely Madruga [a,*]

[a] Post-Graduate Program in Food Science and Technology, Department of Food Engineering, Technology Centre, Federal University of Paraiba, 58051-900 Joao Pessoa, Paraiba, Brazil
[b] Institute of Food Technology (ITAL), Applied Chemistry and Nutrition Center, 13073-001 Campinas, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 11 April 2015
Received in revised form 14 September 2015
Accepted 16 September 2015
Available online 21 September 2015

Keywords:
Cheese
Proteolysis
"Coalho"
Goat cheeses
Free amino acids

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* (BB 12); 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-s_2 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, α_{s1}-casein, as well as structural differences in α-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 (Albenzio & 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 10^7 CFU) (Garcia, Oliveira, Queiroga, Machado, & Souza, 2012; Madureira et al., 2005; Oliveira et al., 2012; Santos et al., 2012). This quantity (10^7 CFU) is the minimum number of bacteria

* Corresponding author.
E-mail address: msmadruga@uol.com.br (M.S. Madruga).

http://dx.doi.org/10.1016/j.foodchem.2015.09.066
0308-8146/

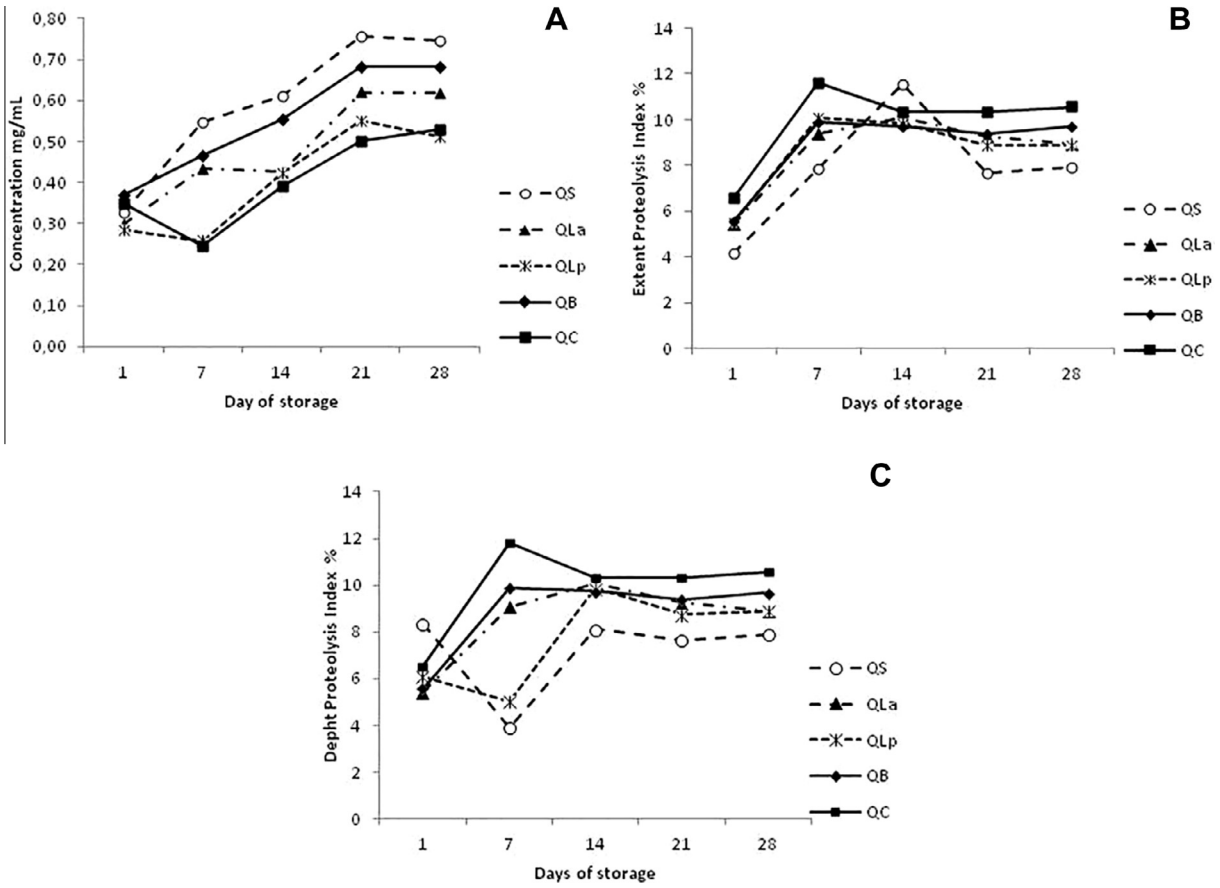

Fig. 1. (A) – Soluble protein (mg mL^{-1}); (B) – extent of proteolysis index (%); (C) – depth of proteolysis index (%) of goat "coalho" cheeses supplemented with probiotic lactic acid bacteria and the starter culture over 28 days of storage at 10 °C ± 2 °C. QS – with starter culture; QLA – with *Lactobacillus acidophilus*; QLP – with *Lactobacillus paracasei*; QB – with *Bifidobacterium lactis*; QC – with co-culture.

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 (Modzelewska-Kapituła, Kobukowski, & Kłebukowska, 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, Ribbera, & 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

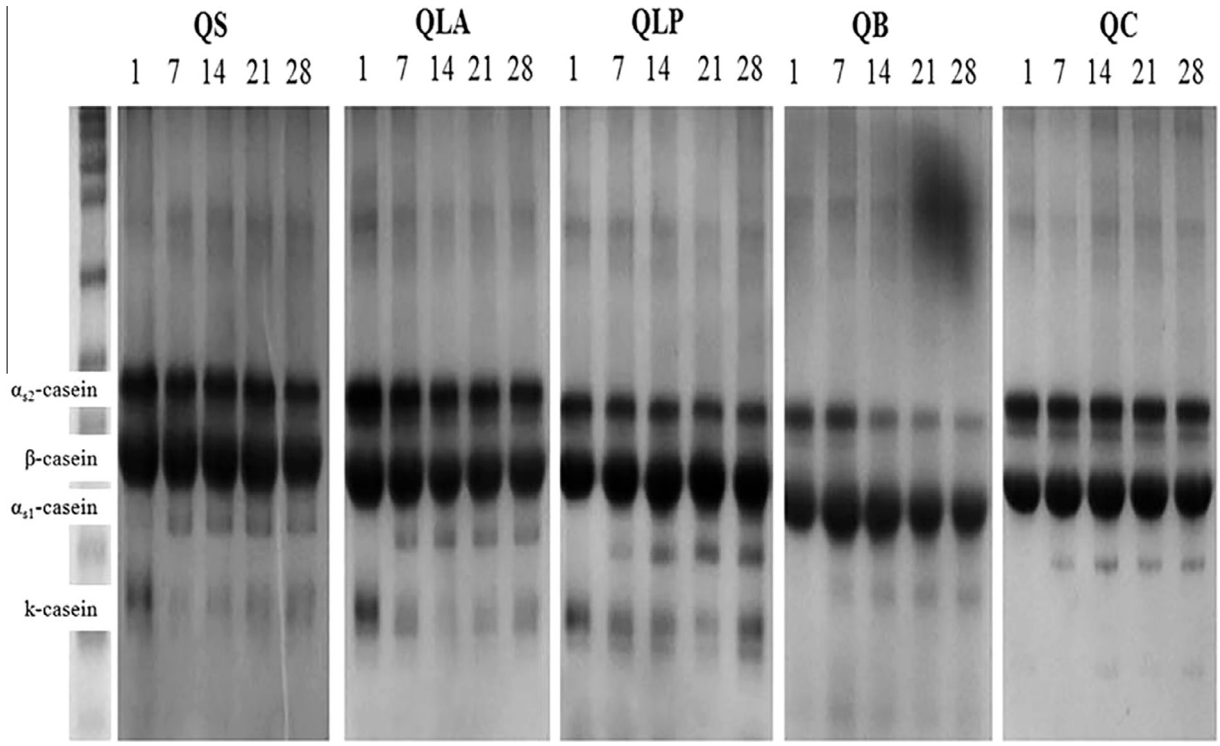

Fig. 2. Protein fractions of goat "coalho" cheese supplemented with probiotic lactic acid bacteria and starter culture over 28 days of storage at 10 °C ± 2 °C. QS – with starter culture; QLA – with *Lactobacillus acidophilus*; QLP – with *Lactobacillus paracasei*; QB – with *Bifidobacterium lactis*; QC – with co-culture.

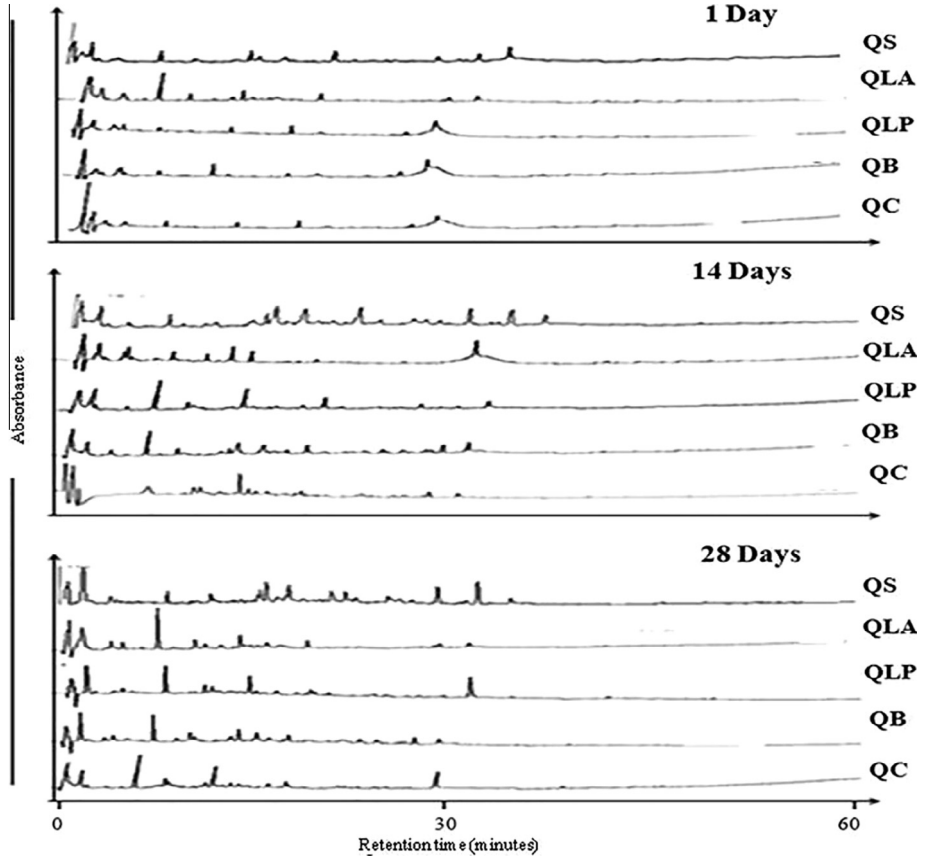

Fig. 3. Reversed-phase HPLC of goat "coalho" cheeses supplemented with probiotic lactic acid bacteria and starter culture over 28 days of storage at 10 °C ± 2 °C. QS – with starter culture; QLA – with *Lactobacillus acidophilus*; QLP – with *Lactobacillus paracasei*; QB – with *Bifidobacterium lactis*; QC – with co-culture.

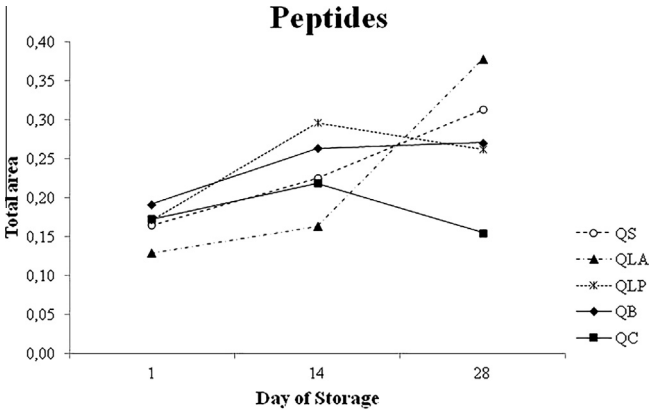

Fig. 4. Total area of reversed-phase HPLC of goat "coalho" cheese supplemented with probiotic lactic acid bacteria during 28 days of storage at 10 °C ± 2 °C. QS – with starter culture; QLA – with *Lactobacillus acidophilus*; QLP – with *Lactobacillus paracasei*; QB – with *Bifidobacterium lactis*; QC – 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 100 mg L^{-1}, being inoculated directly into the vat. Calcium chloride (0.5 mL L^{-1}) and a commercial coagulating agent containing chymosin (0.9 mL L^{-1}) (Chr. Hansen Brazil®, Valinhos, Minas Gerais, Brazil) were also added to the vat.

The vats were maintained at 36 °C until a firm "coalho" was obtained (approximately 40 min). The resulting gel was carefully sliced into cubes (1.5–2.0 cm), and half of the serum was removed for the preparation of the brine (12 g L^{-1} NaCl). The brine was added to the "coalho" and then homogenised. Next, the "coalho" was drained and placed in perforated rectangular moulds (approximately 250 g capacity), which were maintained at 36 °C under pressure for 4 h. The cheeses were then vacuum packaged and stored at 10 °C.

The proteolytic analyses were performed in five seven-day-intervals (1, 7, 14, 21 and 28 days). All the analyses proposed in this study were performed in triplicate.

2.3. Viability of lactic acid bacteria

The counts of the following lactic acid bacteria, as well as the co-culture, were monitored in the cheese: *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*; *L. acidophilus*; *L. paracasei*; and *B. lactis*.

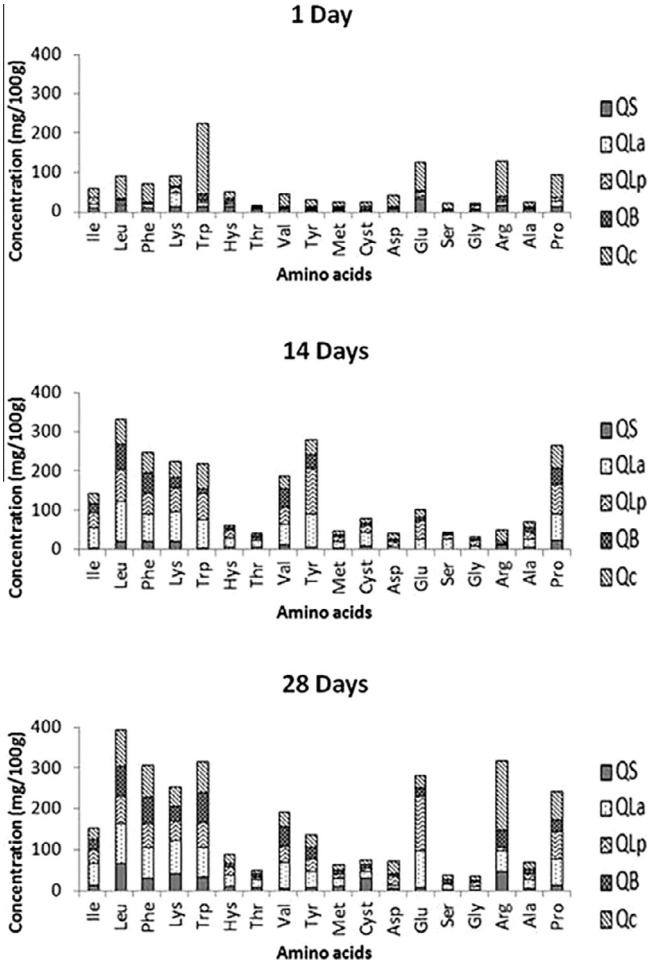

Fig. 5. Free amino acid profile of goat "coalho" cheeses supplemented with probiotic lactic acid bacteria and starter culture at the 1st, 14th and 28th day of storage at 10 °C ± 2 °C. QS – with starter culture; QLA – with *Lactobacillus acidophilus*; QLP – with *Lactobacillus paracasei*; QB – with *Bifidobacterium lactis*; QC – with co-culture.

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

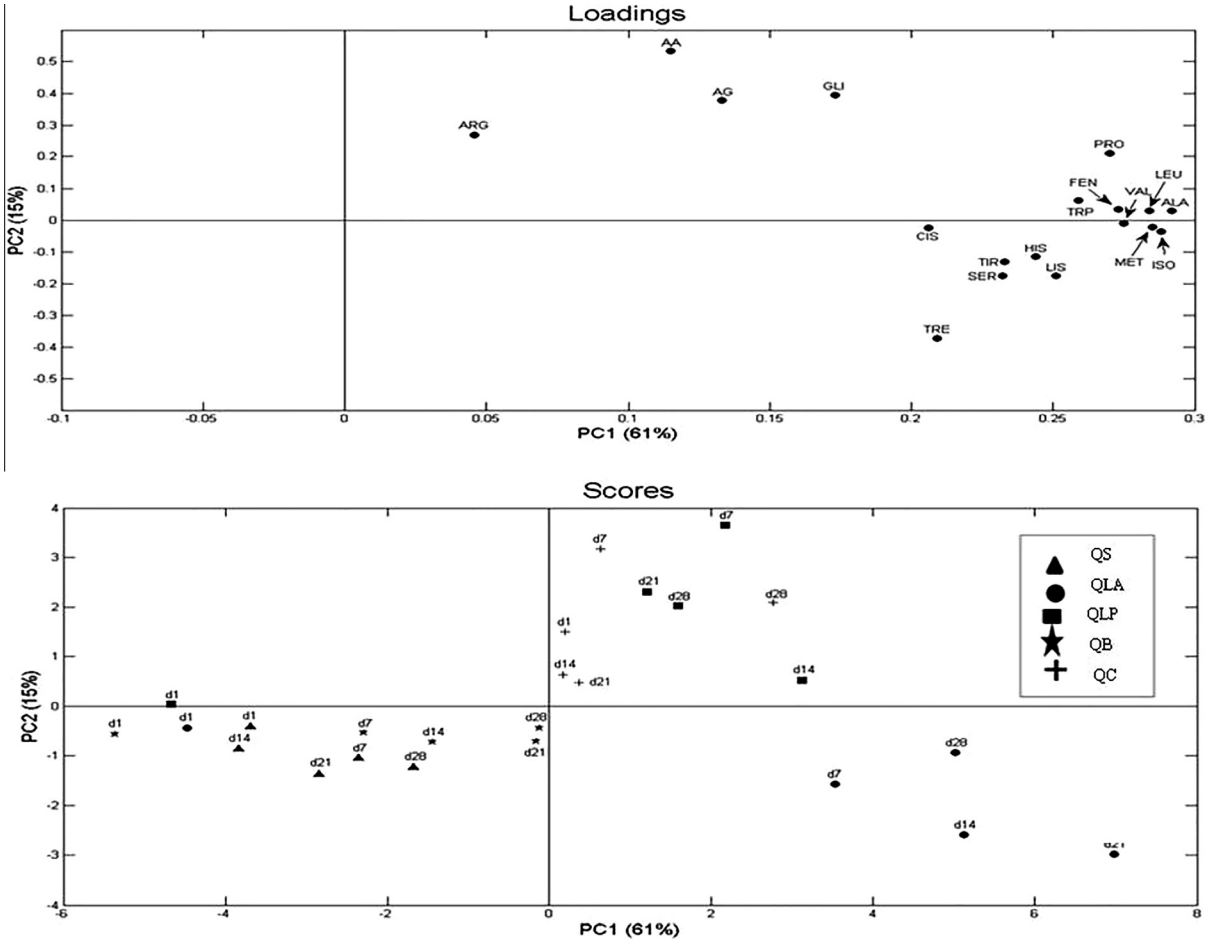

Fig. 6. Principal component analysis of variables divided into two principal components. QS – with starter culture; QLA – with *Lactobacillus acidophilus*; QLP – with *Lactobacillus paracasei*; QB – with *Bifidobacterium lactis*; QC – with co-culture.

equations: EPI = (SN at pH 4.6)/TN (total nitrogen) × 100, and DPI = (TCASN)/TN × 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), Reggianito Argentino 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 α_{s2} 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 α_{s2}-casein is found in the milk of mammals and at high concentrations in goats (Selvaggi, Laudadio, Dario, & Tufarelli, 2014). In turn, α_{s1}-casein may or may not be detected in goat cheese, which is related to the genetics of the goats (Olalla et al., 2009). The low level of this fraction is desirable because it will result in a less allergenic milk. The proportion of α_{s1}-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 α_{s1}-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 $(\alpha)_{s2}$ 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 α_{s2}-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 Strecker degradation reaction, which also leads to the formation of aromatic compounds that affect cheese aroma (Irigoyen, Ortigosa, Juansaras, 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. *cremoris*). 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 α_{s2} 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 α_{s2} 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.

Acknowledgements

The authors would like to thank the National Council for Scientific and Technological Development (CNPq, Brazil) and Coordination for Improvement of Higher Education Personnel (CAPES, Brazil) for the financial support and scholarship Granted to the first author.

References

Albenzio, M., & Santillo, A. (2011). Biochemical characteristics of ewe and goat milk: Effect on the quality of dairy products. *Small Ruminant Research, 101*, 33–40.

Albenzio, M., Santillo, A., Caroprese, M., Ruggieri, D., Napolitano, F., & Sevi, A. (2013). Physicochemical properties of Scamorza ewe milk cheese manufactured with different probiotic cultures. *Journal Dairy of Science, 96*, 2781–2791.

Andreatta, E., Fernandes, A. M., Santos, M. V., Marques, M. C., Gigante, M. L., & Oliveira, C. A. F. (2007). Effects of milk somatic cell count on physical and chemical characteristics of mozzarella cheese. *Australian Journal of Dairy Technology, 62*, 166–170.

AOAC – Association of Official Analytical Chemists. (2010). *Official Methods of Analysis*. Gaithersburg, MD, USA.

Bergamini, C. V., Hynes, E. R., Quiberoni, A., Sauárez, V. B., & Zalazar, C. A. (2005). Probiotic bacteria as adjunct starters: Influence of the addition methodology on their survival in a semi-hard Argentinean cheese. *Food Research International, 38*, 597–604.

Bontinis, T. G., Mallatou, H., Pappa, E. C., Massouras, T. H., & Alichanidis, E. (2012). Study of proteolysis, lipolysis and volatile profile of a traditional Greek goat cheese (Xinotyri) during ripening. *Small Ruminant Research, 105*, 193–201.

Burns, P., Cuffia, F., Milesi, M., Vinderola, G., Meinardi, C., Sabbag, N., & Hynes, E. (2012). Technological and probiotic role of adjunct cultures of non-starter lactobacilli in soft cheeses. *Food Microbiology, 30*, 45–50.

Costa, W. K. A., Souza, E. L., Beltrão-Filho, E. M., Vasconcelos, G. K. V., Gadelha, T. S., Gadelha, C. A. A., ... Magnani, M. (2014). Comparative protein composition analysis of goat milk produced by the alpine and saanen breeds in northeastern Brazil and related antibacterial activities. *PLoS ONE, 9*, 1–9.

De Vuyst, L. (2000). Technology aspects related to the application of functional starter cultures. *Food Technology and Biotechnology, 38*, 105–112.

Egito, A. S., Miclo, L., López, C., Adam, A., Girardet, J. M., & Gaillard, J. L. (2002). Separation and characterization of mares' milk alpha(s1)-, beta-, kappa-caseins,

gamma-casein-like, and proteose peptone component 5-like peptides. *Journal of Dairy Science, 85*, 697–706.
Escobar, M. C., Van Tassell, M. L., Martínez-Busto, F., Singh, M., Castaño-Tostado, E., Amaya-Llano, S. L., & Miller, M. J. (2012). Characterization of a panela cheese with added probiotics and fava bean starch. *Journal of Dairy Science, 95*, 2779–2787.
FDA – Food and Drug Administration (1992). *Bacteriological analytical manual* (7th ed.). Arlington, VA, USA: AOAC International.
Garcia, E. F., Oliveira, M. E. G., Queiroga, R. C. R. E., Machado, T. A. D., & Souza, E. L. (2012). Development and quality of a Brazilian semi-hard goat cheese (coalho) with added probiotic lactic acid bacteria. *International Journal of Food Science and Nutrition, 63*, 947–956.
Gomes, A. A., Braga, S. P., Cruz, A. G., Cadena, R. S., Lollo, P. C. B., Carvalho, C., ... Bolini, H. M. A. (2011). Effect of the inoculation level of *Lactobacillus acidophilus* in probiotic cheese on the physicochemical features and sensory performance compared with commercial cheeses. *Journal of Dairy Science, 94*(10), 4777–4786.
Hagen, S. R., Frost, B., & Augustin, J. (1989). Pre column phenylisothiocyanate derivatization and liquid chromatography of amino acids in food. *Journal of the Association of Official Analytical Chemists, 72*, 912–916.
Hayaloglu, A. A., Tolu, C., & Yasar, K. (2013). Characterizing volatile compounds and proteolysis in Gokceada artisanal goat cheese. *Small Ruminant Research, 113*, 187–194.
Iličić, M. D., Milanović, S. D., Carić, M. D., Kanurić, K. G., Vukić, V. R., Hrnjez, D. V., & Ranogajec, M. I. (2012). Volatile compounds of functional dairy products. *Acta Periodica Technologica, 43*, 11–19.
Irigoyen, A., Ortigosa, M., Juansaras, I., Oneca, M., & Torre, P. (2007). Influence of an adjunct culture of *Lactobacillus* on the free amino acids and volatile compounds in a Roncal-type ewe's-milk cheese. *Food Chemistry, 100*, 71–80.
Kirmaci, H. A., Hayaloglu, A. A., Ozer, H. B., Atasoy, A. F., & Turkoglu, H. (2014). Evolution of proteolysis in Urfa cheese made from ewe's milk by wild type starter culture systems. *Small Ruminant Research, 119*, 120–129.
Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680.
Lollo, P. C. B., Cruz, A. G., Morato, P. N., Moura, C. S., Carvalho-Silva, L. B., Oliveira, C. A. F., ... Amaya-Farfan, J. (2012). Probiotic cheese attenuates exercise-induced immune suppression in Wistar rats. *Journal of Dairy Science, 95*, 3549–3558.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal Biological Chemistry, 193*, 265–275.
Madureira, A. R., Pereira, C. I., Truszkowska, K., Gomes, A. M., Pintado, M. E., & Malcata, F. X. (2005). Survival of probiotic bacteria in a whey cheese vector submitted to environmental conditions prevailing in the gastrointestinal tract. *International Dairy Journal, 15*, 921–927.
Michaelidou, A., Katsiari, M. C., Voutsinas, L. P., Kondyli, E., & Alichanidis, E. (2003). Effect of commercial adjunct cultures on proteolysis in low-fat Kefalograviera-type cheese. *International Dairy Journal, 13*, 743–753.
Modzelewska-Kapituła, M., Kobukowski, J. A. N., & Kłebukowska, L. (2010). The influence of feeding diets containing white cheese, produced with prebiotics and the potentially probiotic *Lactobacillus plantarum* strain, on the gastrointestinal microflora of rats. *Czech Journal of Food Sciences, 28*, 139–145.
Olalla, M., Ruiz-Ló Pez, M. D., Navarro, M., Artacho, R., Cabrera, C., Giménez, R., ... Mingorance, R. (2009). Nitrogen fractions of Andalusian goat milk compared to similar types of commercial milk. *Food Chemistry, 113*, 835–838.
Oliveira, E. M. G., Garcia, E. F., Queiroga, R. C. R. E., & Souza, E. L. (2012). Technological, physicochemical and sensory characteristics of a Brazilian semi-hard goat cheese (coalho) with added probiotic lactic acid bacteria. *Scientia Agricola, 69*, 370–379.
Potje, S. R., Munhoz, F. C., Perassa, L. A., Graton, M. E., Pereira, A. A. F., Nakamune, A. C. M. S., ... Antoniali, C. (2014). Mechanisms underlying the hypotensive and vasodilator effects of Ru (terpy) (bdq) NO^{3+}, a nitric oxide donor, differ between normotensive and spontaneously hypertensive rats. *European Journal of Pharmacology, 741*, 222–229.
Queiroga, R. C. R. E., Santos, B. M., Gomes, A. M. P., Monteiro, M. J., Teixeira, S. M., Souza, E. L., ... Pintado, M. M. E. (2013). Nutritional, textural and sensory properties of coalho cheese made of goats' milk, cows' milk and their mixture. *LWT – Food Science and Technology, 50*, 538–544.
Randazzo, C. L., Pitino, I., Ribbera, A., & Caggia, C. (2010). Pecorino Crotonese cheese: Study of bacterial population and flavour compounds. *Food Microbiology, 27*, 363–374.
Rodrigues, D., Rocha-Santos, T. A. P., Pereira, C. I., Gomes, A. M., Malcata, X., & Freitas, A. C. (2011). The potential effect of FOS and inulin upon probiotic bacterium performance in curdled milk matrices. *LWT – Food Science and Technology, 44*, 100–108.
Santos, K. M. O., Bomfim, M. A. D., Vieira, A. D. S., Benevides, S. D., Saad, S. M. I., Buriti, F. C. A., & Egito, A. S. (2012). Probiotic goat coalho cheese naturally enriched in conjugated linoleic acid as a vehicle for *Lactobacillus acidophilus* and beneficial fatty acids. *International Dairy Journal, 24*, 107–112.
Selvaggi, M., Laudadio, V., Dario, C., & Tufarelli, V. (2014). Major proteins in goat milk: An updated overview on genetic variability. *Molecular Biology Reports, 41*, 1035–1048.
Silva, F. A. S., & Azevedo, C. A. V. (2009). *Principal components analysis in the software Assistat – Statistical attendance World Congress on Computers in Agriculture* (Vol. 7). Reno-NV-USA: American Society of Agricultural and Biological Engineers.
Silva, R. A., Lima, M. S. F., Viana, J. B. M., Bezerra, V. S., Pimentel, M. C. B., Porto, A. L. F., ... Lima Filho, J. L. (2012). Can artisanal "coalho" cheese from northeastern Brazil be used as a functional food? *Food Chemistry, 135*, 1533–1538.
Sinz, Q., & Schwab, W. (2012). Metabolism of amino acids, dipeptides and tetrapeptides by *Lactobacillus sakei*. *Food Microbiology, 29*, 215–223.
Steele, J., Broadbent, J., & Kok, J. (2013). Perspectives on the contribution of lactic acid bacteria to cheese flavor development. *Current Opinion in Biotechnology, 24*, 135–141.
Talwalkar, A., Miller, C. W., Kailasapathy, K., & Nguyen, M. H. (2004). Effect of packaging materials and dissolved oxygen on the survival of probiotic bacteria in yoghurt. *International Journal of Food Science & Technology, 39*, 605–611.
White, J. A., Hart, R. J., & Fry, J. C. (1986). An evaluation of the Waters Pico-Tag system for the amino-acid analysis of food materials. *Journal of Automatic Chemistry of Clinical Laboratory Automation, 8*, 170–177.
Wolf, I. V., Perotti, M. C., Bernal, S. M., & Zalazar, C. A. (2010). Study of the chemical composition, proteolysis, lipolysis and volatile compounds profile of commercial Reggianito Argentino cheese: Characterization of Reggianito Argentino cheese. *Food Research International, 43*, 1204–1211.