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A new milk-clotting enzyme produced by *Bacillus* sp. P45 applied in cream cheese development

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ABSTRACT

The growing demand for natural coagulants led to an increased necessity for rennet substitutes, promoting a search for new sources of proteases with coagulant properties. The aim of this study was to investigate the application of a bacterial enzyme as a novel milk-clotting protease in the development of cream cheese enriched with chia and quinoa flour. At the concentration of 30 mg/mL, the milk-clotting strength was similar to that observed for commercial chymosin, demonstrating the enzyme ability to catalyze the hydrolysis of milk casein. The cheese developed showed high water retention (≥99.0%) and consequently low syneresis process. The results indicate that the product made using the enzyme showed adequate sanitary conditions and technological characteristics indicated that the product is highly stable and viable.

1. Introduction

Obtaining natural milk coagulants is a challenge since rennet availability is limited compared with the increasing demand of the dairy industry. Natural coagulants of animal origin are expensive, and their consumption has been restricted due to religious or dietary reasons. This scenario has led to a growing demand for novel rennet substitutes, promoting a search for new sources of proteases with coagulant properties (Ahmed, Morishima, Babiker, & Mori, 2009; Jacob, Jaros, & Rohm, 2011; Mazorra-Manzano et al., 2013).

Different proteases have been used for this purpose, including those extracted from vegetables (Corrons, Bertucci, Liggieri, Lopez, & Bruno, 2012; Galán, Cabezas, & Fernández-Salguero, 2012) and animals (Rolet-Repecaud et al., 2013; Shamtsyan, Dmitriyeva, Kolesnikov, & Denisova, 2014; Trujillo, Guamis, Laencina, & Lopez, 2000), but their applications may be limited by factors, such as cultivation and climatic variations, which can affect their production and supply.

Thus, microbial enzymes are attractive and have been tested in milk coagulation (Daroit et al., 2012). *Bacillus* sp. P45, isolated from the intestine of Jaraqui fish (*Piaractus mesopotamicus*) originating from the Amazon basin (Sirtori, Cladera-Olivera, Lorenzini, Tsai, & Brandelli, 2006), produces an enzyme that presents great potential for protein hydrolysis (Daroit, Correa, & Brandelli, 2009). A keratinolytic protease was purified and characterized as a subtilisin-like serine protease of about 26 kDa that hydrolyses casein at high rates (Daroit, Corrêa, Segalin, & Brandelli, 2010). This enzyme also produces bioactive hydrolysates during proteolysis of ovine casein (Daroit et al., 2012). Therefore, this protease may be useful for development of dairy products through milk-clotting processes.

Dairy products enriched with bioactive compounds have attracted increased consumer interest. Quinoa (*Chenopodium quinoa Willd.*) is a seed recognized for its high content of essential amino acids, protein, fiber and minerals (Madl, Sterk, & Mittelbach, 2006; Nsimba, Kikuzaki, & Konishi, 2008). Also, chia (*Salvia hispanica* L.) presents high contents of protein, fiber (Capitani, Spotorno, Nolasco, & Tomas, 2012; Marineli et al., 2014) and elevated amount of α-linolenic acids (Ayerza & Coates, 2011). Both seeds have a high antioxidant activity (Capitani et al., 2012), associated with protection against lipid oxidation, inflammatory processes, cancer and other diseases related to oxidative stress (Gawlik-Dziki et al., 2013). This suggests that quinoa and chia can be useful as functional ingredients in food

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formulations (Marineli et al., 2014).

The aim of this study was to investigate the milk-clotting ability of a protease obtained from *Bacillus* sp. P45 and apply the enzyme in the development of cream cheese enriched with chia and quinoa flour.

2. Materials and methods

2.1. Microorganism, inoculum, cultivation and enzyme purification

The enzyme was produced by submerged cultivation of *Bacillus* sp. P45 using feather meal as a substrate (Daroit, Correa, & Brandelli, 2011), and then purified using aqueous two-phase system integrated into the diafiltration process (Sala et al., 2014). The enzyme preparation containing the partially purified protease with molecular mass around 26–28 kDa (Daroit et al., 2010; Sala et al., 2014) was lyophilized and stored at 4 °C for use in later steps.

2.2. Milk-clotting enzyme activity (MCA)

The MCA was examined by the method of Berridge (1952) using the crude enzyme, purified enzyme, and commercial chymosin (Agrolac Parana®) as standard. Milk aliquots were incubated at 30 °C and 1 mL of the enzyme solution (10 to 50 mg/mL), was added. The clotting time was determined from the clot formation in the tube wall. MCA is defined as the amount of enzyme that clots 10 mL of reconstituted skim milk in 100 seconds at 30 °C (Eq. (1)). The correlation between the inverse of the enzyme concentration and the MCA was done to predict the coagulation time and the coagulant activity of the enzymes.

$$\mathrm{MCA} = \frac{10 \cdot \text{volume of milk}}{\text{clotting time (s)} \cdot \text{coagulant volume}} \quad (1)$$

2.3. Cream cheese development

The cream cheese enriched with chia and quinoa flour was produced in the Dairy Institute of the UNL (Santa Fé, Argentina) using the purified protease obtained from *Bacillus* sp. P45 that was standardized in relation to the milk-clotting activity of commercial chymosin enzyme. The cheese was obtained by mixing milk powder, cream and water at 50 °C for 5 min (Fig. 1). Then, the milk was pasteurized (75 °C/15 s) and homogenized (150 atm). Milk was pasteurized again (75 °C/15 s), adjusted to 50 °C and added espina corona, chia and quinoa flour, whey protein concentrate, milk powder and potassium sorbate. The mixture was stirred for 10 min, pasteurized (75 °C/15 s) and cooled (45 °C) for calcium citrate addition. Milk was placed in a thermostatic bath at 40 °C and a starter culture containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, and the purified enzyme were added. After the clotting process, the curd mass was homogenized (50 atm) and cooled (15 °C). The formulations were stored in sealed packages at 4 °C. The product was evaluated in relation to pH and acidity (every 5 days), texture profile and water retention (days 1, 13 and 25 of storage), water activity and total solids (13th day of storage). The chemical composition and microbiological analyses were performed on the 25th day of storage. All samples were made by the same process, however, presented different relative concentrations of cream, chia and quinoa flour (Table 1), as previously established by the research group.

2.4. Cream cheese profile

2.4.1. pH and acidity determination during cream cheese storage

The pH was measured potentiometrically and the acidity by direct titration with 0.1 mol/L NaOH (AOAC, 2005). The results were determined as Dornic degrees and converted to lactic acid concentration (mol/L).

2.4.2. Water retention and water activity

Water retention (%WR) analyses were performed during samples storage by centrifugation and gravimetry. The %WR was determined by centrifugation of the samples (1000× *g*, 20 min, at 4 °C) stored at 4 °C in suitable containers containing 50 g of cheese. After centrifugation, the supernatant was drained and weighed to determine the water retention (Eq. (2)).

$$\%WR = 100 - \Big((\textit{Initial weight} - \textit{final weight})/(\textit{inital weight})\Big) \times 100 \quad (2)$$

The %WR by gravity was determined by weighing the tubes containing the samples stored under the same conditions. The supernatant fluids present on the surface were drained and weighed to determine the parameters. The water activity was determined at 2 °C using Aqua Lab CX-2T equipment.

2.4.3. Texture profile

The texture profile was determined using an Instron Bluehill® texturometer from a stress curve in Newton (N) versus time (s). The following mechanical properties were determined: hardness, adhesiveness, springiness, cohesiveness, gumminess, chewiness and resilience. The parameters used in the tests were double penetration of 30 mm into the samples, speed penetration of 1 mm/s, bristle of 10 N, penetrometer diameter of 12 mm and cylinder diameter of 36 mm at 10 °C (Pons & Fiszman, 1996; Santini et al., 2007).

2.4.4. Chemical and microbiological analyses

The chemical composition (moisture, ash, protein, fiber and lipids) was determined by standard procedures and carbohydrates were calculated by difference (AOAC, 2005). Caloric values were calculated using conversion factors based on the chemical composition from the sum of the protein content × 4, lipids × 9 and carbohydrates × 4 (Horwitz, 1997). Microbiological analyses were performed for *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes*, total and fecal coliforms, aerobic mesophilic, molds and yeasts, following standard protocols (Doores, Salfinger, & Tortorello, 2013).

2.5. Statistical analysis

Data were subjected to analysis of variance to detect significant differences between treatments by Tukey's test. Differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Milk-clotting activity

Both crude and purified enzyme at the concentration of 30 mg/mL showed similar milk-clotting capability to that observed for commercial coagulant (Table 2), demonstrating the potential to be used as an alternative coagulant. The results indicated that MCA was dependent of the enzyme concentration; the milk-clotting time decreased as the enzyme concentration increased, similar to that observed for plant enzymes (Ahmed et al., 2009; Beka et al., 2014; Chazarra, Sidrach, Lopez-Molina, & Rodriguez-Lopez, 2007). A clear linear correlation between the inverse of the enzyme

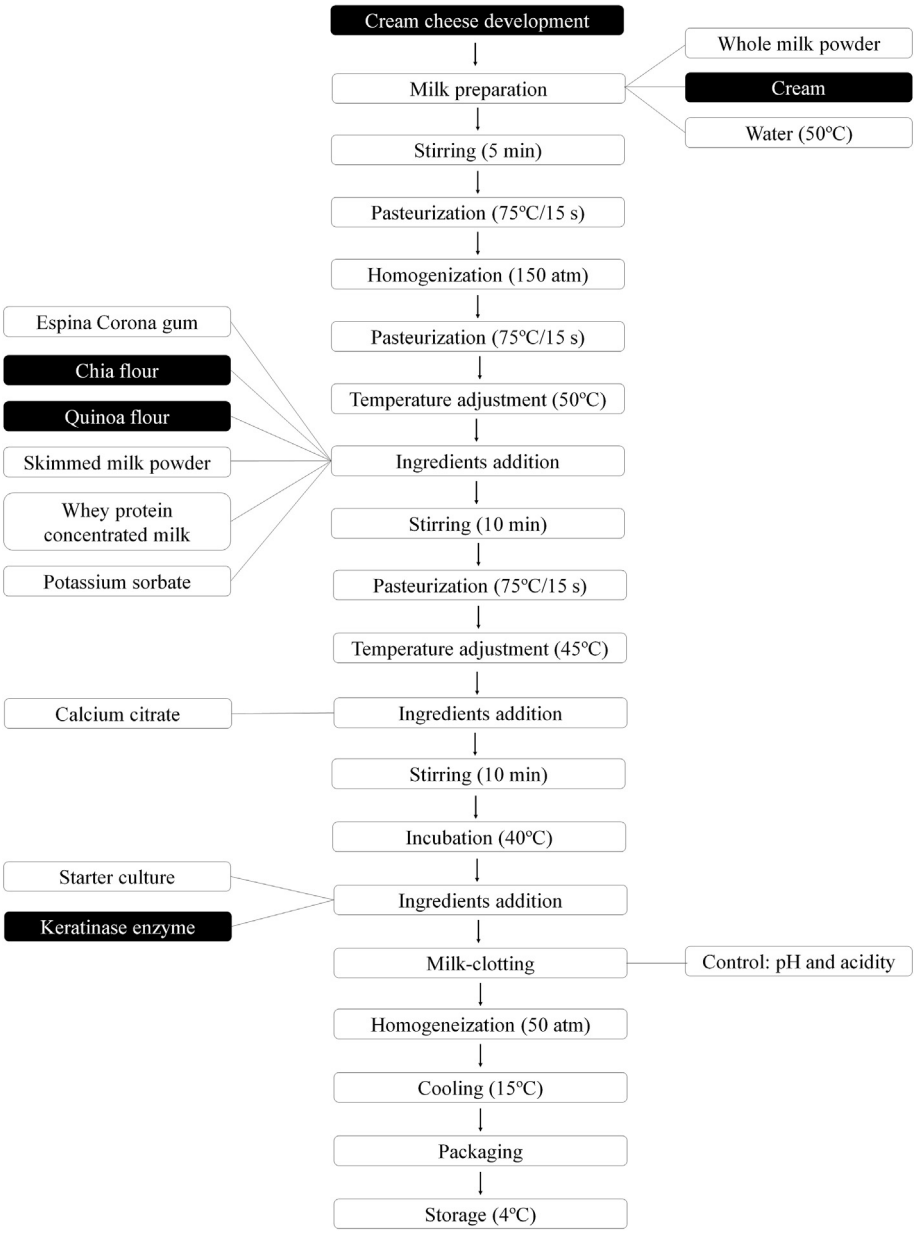

Fig. 1. Flowchart of cream cheese development.

Table 1
Cream cheese formulations made from purified enzyme.

Ingredients (%)	Formulations				
	1	2	3	4	5
Cream	6.0	10.0	6.0	10.0	8.0
Chia flour	4.0	4.0	1.0	1.0	2.5
Quinoa flour	1.0	1.0	4.0	4.0	2.5

concentration and the MCA values was verified for the crude and the purified enzyme (Crude enzyme = 36.1 + 2034.9*x, $r^2 = 0.9994$; Purified enzyme = 23.9 + 2390.6*x, $r^2 = 0.9995$), thus making it possible to predict the coagulation time and the coagulant activity of the enzyme. The clots formed by the three enzymes (crude, purified and commercial) showed the same characteristics: stability and transparent whey exudate. These results showed that both the purified and crude enzyme could be used as alternative milk coagulants.

Table 2
Milk-clotting activity of crude, purified and commercial enzyme.

Enzyme	(mg/mL)	Clotting time (s)	Chymosin unit
Crude	10	239 ± 7.2^{e}	0.42 ± 1.10^{-2e}
	20	140 ± 2.0^{d}	0.71 ± 1.10^{-2d}
	30	105 ± 1.7c	0.95 ± 2.10^{-2c}
	40	85 ± 1.5^{b}	1.17 ± 2.10^{-2b}
	50	76 ± 2.6^{a}	1.32 ± 5.10^{-2a}
Purified	10	264 ± 4^{e}	0.38 ± 5.10^{-3e}
	20	141 ± 3^{d}	0.71 ± 0.01^{d}
	30	105 ± 2^{c}	0.96 ± 0.02^{c}
	40	82 ± 2^{b}	1.22 ± 0.02^{b}
	50	74 ± 2^{a}	1.36 ± 0.03^{a}
Commercial	1 mL	106 ± 1^{c}	0.96 ± 0.01^{c}

The statistical analysis of the means was conducted using Tukey's test. The same letters in the columns represent no significant differences at the significance level of 5%.

The optimal coagulant activity for dairy product development is crucial because of its influence on the technological properties of the product, including the texture parameters (Børsting, Stallknecht, Vogensen, & Ardö, 2015) and the sensory attributes (Galán, Prados, Pino, Tejada, & Fernández-Salguero, 2008). Despite the similarity in the MCA, the use of purified enzyme could be advantageous since that preliminary sensory tests, conducted by trained evaluators, indicated that the crude enzyme caused intense acetic odor and brown coloration, affecting the color and aroma of the final product, and therefore, consumer acceptance. Thus, the purified enzyme was chosen to be applied in the cream cheese development.

3.2. Cream cheese profile

3.2.1. pH and acidity

A decline of pH and proportional increase in lactic acid production was observed in all samples (Fig. 2). The pH ranged from 6.6 to 6.3 at the beginning to 5.3 at the end of fermentation process. The lactic acid concentration ranged from 0.02 to 0.03 at the initial phase, reaching 0.06–0.1 mol/L at the end of fermentation process, lasting approximately 5 h.

The pH reduction and increase in acidity occurs due to lactic acid fermentation that is initiated when the starter culture is added. The microorganisms perform the hydrolysis of lactose into glucose and galactose, and the monosaccharides are subsequently transformed into lactic acid. The lactic acid production causes a drop in the pH during fermentation and storage because most of the enzymes remain active and the microorganisms remain viable. However, this effect is less marked during the storage, as low temperatures are used (Rojas-Castro, Chacón-Villalobos, & Pineda-Castro, 2007). In addition to provide specific sensory characteristics, pH reduction is important to prevent the growth of pathogenic bacteria. The pH variation during storage also depends on the buffering capacity of the cheese, which is related to the amount of protein and minerals, ammonium formation, and/or lactic acid catabolism (Lawrence, Heap, & Gilles, 1984; Merheb-Dini, Garcia, Penna, Gomes, & da Silva, 2012).

A reduction in pH (Fig. 3A) and an increase of acidity (Fig. 3B) was observed in all formulations during the storage period. During the first ten days, the pH decreased and lactic acid production was more pronounced than in subsequent periods, with a tendency to stabilize at the end of storage. This could be due to several factors, including the inhibition of the enzymatic activity present in the lactic culture, the viability of the microbial load, represented by the starter culture employed, and the lactose depletion combined with low temperature (Rojas-Castro et al., 2007).

The pH stabilizing effect at the end of the storage is a natural and desirable phenomenon during the development of fermented dairy products and has been previously described in several studies. Additionally, the pH values and acidity observed in this study are comparable with those reported by other authors (Deegan, Holopainen, McSweeney, Alatossava, & Tuorila, 2014; Olmedo,

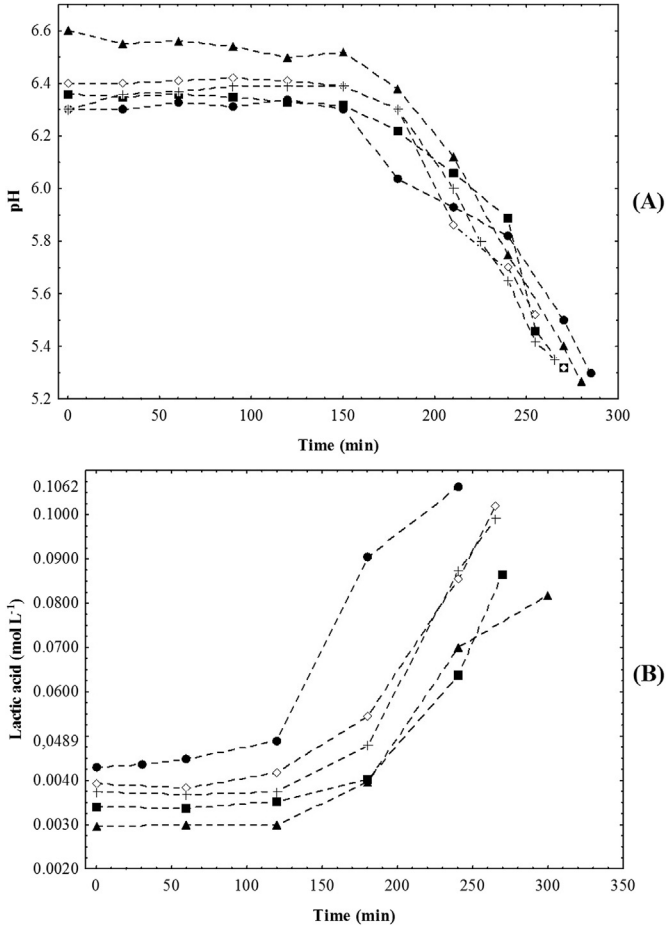

Fig. 2. pH (A) and acidity (B) of the formulations prepared from purified enzyme during the fermentation process. (●)1, (■)2, (◊)3, (▲)4 and (+)5.

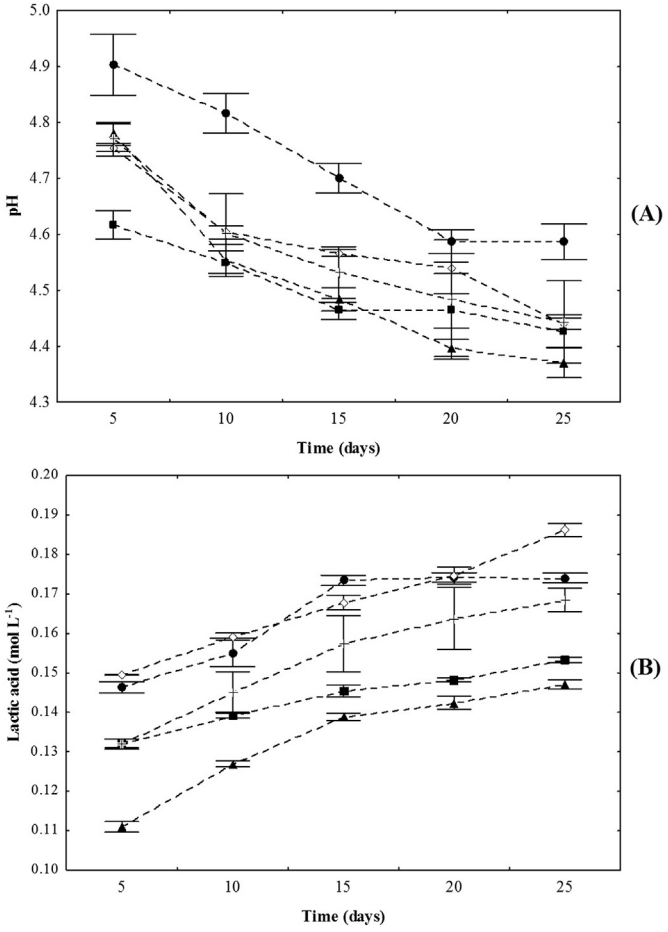

Fig. 3. pH (A) and acidity (B) of the five formulations prepared from purified keratinase enzyme during the storage by 25 days. (●)1, (■)2, (◊)3, (▲)4 and (+)5.

Nepote, & Grosso, 2013) and are consistent with those typically expected for this product.

3.2.2. Water activity and chemical composition

The chemical composition of the formulations (Table 3) was similar to other cream cheeses described in the literature (Souza, Carneiro, Pinto, Souza, & Stephani, 2012; Zulkurnain, Goh, Karim, & Liong, 2008). The lipid concentration was proportional to the content of cream added during the development and was significantly higher for formulations 2, 4 and 5. These formulations presented 10% lipids, while samples 1 and 3 showed 7.0 and 5.5% lipids, respectively. The moisture content of all samples was greater than 70.0%, and is characterized by specific legislation (ANVISA, 2001) as a product of very high humidity.

The a_w values were similar for all samples, regardless the amount of cream, chia or quinoa flour used. The a_w determination is important because it is related to the available water, which can be used for deteriorative reactions or microbial metabolism (Ostrowska-Ligęza & Lenart, 2015). The a_w values, approximately 0.96, indicate that the product would be susceptible to most bacteria and fungi, but the acidic pH of the cheese protects against several pathogenic bacteria.

The fiber content varied from 3.00 to 4.96% and formulation 3 showed the highest value. The US Food and Drug Administration allows foods to be labeled as 'a good source of fiber' or 'high fibre' if they contain more than 2.5 g or 5.0 g of dietary fibre per serving, respectively (Jin, Hsien, & Huff, 1994). Development of products with high sensory acceptance and high fiber contents could minimize dietary disturbances and stimulate a healthy lifestyle. The increase in life expectancy has generated a great interest in foods that are rich in fibers, which may prevent some diseases such as diabetes mellitus, cardiovascular diseases, obesity and colon cancer (Tharanathan & Mahadevamma, 2003).

The protein content ranged from 5.4 to 7.4% and was significantly higher for formulations 1 and 3, with lower quantities of cream and lower amounts of chia and quinoa flour, respectively. The incorporation of these products may be indicated as a protein source, especially with regard to the contribution of the essential amino acids contained in the seeds of chia and quinoa (Madl et al., 2006).

3.2.3. Water retention

Regarding the water retention, no significant differences were observed among the formulations for the initial period of storage. The values were identical when evaluated by gravity (Fig. 4A) and very similar by centrifugation (Fig. 4B).

A decrease in the water retention causes the syneresis process, which refers to the serum that is released from the gel structure of the cheese and accumulates on the surface. An increase in the syneresis process was observed in the 13th day of storage, with a significant elevation ($P \leq 0.05$) during storage for 25 days, which can be verified by reduction of water retention values (Fig. 4). This occurrence was more evident when the samples were evaluated by centrifugation, indicating a greater water release from the cheese matrix.

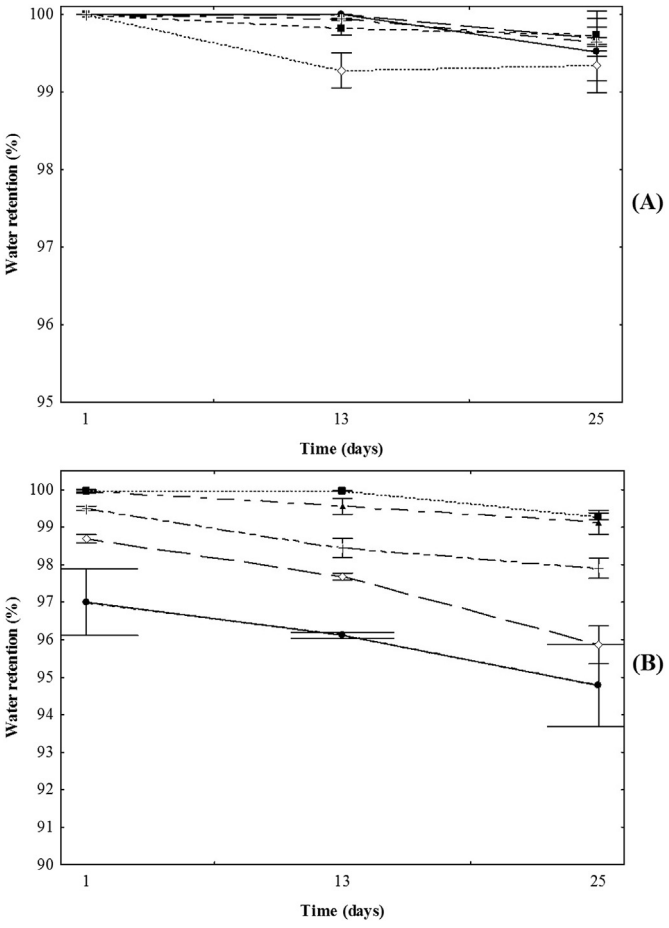

Fig. 4. Water retention (%) determined by gravity (A) and centrifugation (B) of the five formulation: (●) 1, (■)2, (◊)3, (▲)4 and (+)5.

Despite the increase in water release, the syneresis process verified by gravity analysis that simulates the product exposure under normal storage conditions was practically null, indicating a low risk of syneresis process and consequently absence of serum on the cheese surface. This allows us to assume that the enzyme application, in combination with the components used in the cheese, produces clots with high stability and water retention. The

Table 3
Chemical composition of cream cheese developed.

Components (%)[a]	Formulations				
	1	2	3	4	5
Moisture	72.24 ± 0.46^{bc}	73.42 ± 0.32^{ab}	72.04 ± 0.52^{c}	74.2 ± 0.51^{a}	73.01 ± 0.1^{abc}
Protein	7.4 ± 0.21^{a}	5.7 ± 0.37^{c}	6.9 ± 0.62^{ab}	5.4 ± 0.42^{c}	6.2 ± 0.27^{bc}
Lipids	7.0 ± 0.72^{b}	10.0 ± 0.47^{a}	5.5 ± 0.62^{b}	10.0 ± 0.25^{a}	10.0 ± 0.82^{a}
Fiber	3.54 ± 0.32^{b}	3.0 ± 0.81^{b}	4.96 ± 0.09^{a}	3.44 ± 0.07^{b}	4.08 ± 0.68^{ab}
Ash	1.69 ± 0.02^{a}	1.29 ± 0.04^{d}	1.59 ± 0.02^{b}	1.23 ± 0.00^{d}	1.37 ± 0.03^{cd}
Carbohydrate	8.13	6.59	9.01	5.7	5.34
Water activity	0.962 ± 0.004^{a}	0.962 ± 0.002^{a}	0.958 ± 0.001^{a}	0.962 ± 0.004^{a}	0.961 ± 0.002^{a}
Calories (kcal)	125.12	139.16	113.14	134.4	136.16

The statistical analysis of the means employed Tukey's test, and different letters in the same row represent significant differences at the significance level of 5%.
[a] Except for water activity and caloric value.

stability may also be favored by the addition of quinoa and chia because they also present a high water retention capacity (Bhargava, Shukla, & Ohri, 2006; Capitani et al., 2012), especially due to the high protein content. In addition, stabilizers used in the samples, such as espina corona gum tend to "bind" water in the gel structure, generally improving the hydration of proteins. Despite the reduction of the %WR values during storage ($P < 0.05$), the lower value obtained with respect to gravity (99.3%) was insignificant and does not affects this quality attribute. The high protein and fat contents contribute to a stable structure, retaining water during storage (Zulkurnain et al., 2008). The formulations 2 and 4, with the highest cream concentration, showed the highest water retention when measured by centrifugation, and, consequently, a lower syneresis process during the storage, mainly due to the emulsifying properties (Mateo et al., 2009). The opposite was also observed; samples with less cream addition showed the greatest water release from the cheese matrix.

3.2.4. Texture profile

The texture profile of the formulations was evaluated in different storage times (Table 4). In general, all parameters showed significant differences ($P < 0.05$) as a function of time and the formulations studied. The elasticity was the only parameter that showed no significant difference, suggesting no change in the recovery of initial cheese dimensions after the removal of the deforming force (Santini et al., 2007).

Formulation 3, with the highest quinoa and less cream content, had the highest hardness. This suggests that the high capacity of quinoa to absorb water, combined with a lower fat concentration, which is responsible for creaminess and smoothness, can contribute to a greater resistance to deformation (Santini et al., 2007). Formulations 1, 2 and 5 showed an increase in hardness on the 13th day of storage, while formulations 3 and 4 showed an increase in hardness on the 25th day of storage.

The adhesiveness was higher for samples with higher quinoa contents, indicating the formation of a resistant structure that requires a greater force for removing the cheese from the contact surface of the mouth (Bryant, Ustunol, & Steffe, 1995; Karaman & Akalin, 2013). Formulations 1, 2 and 3 showed no significant variation in adhesiveness during the 25 days of storage. However, formulations 4 and 5 showed an increase in adhesiveness on the 13th day of storage.

The cohesiveness was significantly higher for formulation 1, where higher chia and lower cream contents were used. These conditions favored the production of a more elastic gel that requires a greater effort to deform the cream cheese. There was no change in the cohesiveness parameter for formulations 1 and 5. However, this parameter was significantly altered in formulations 2, 3 and 4 on the 25th day of storage.

The gumminess and chewiness showed the same behavior. The highest values were found for samples with higher quinoa contents, demonstrating their influence on the texture profile. Both gumminess and chewiness remained equal in formulation 3 during 25 days of storage. However, there was significant difference for formulations 1, 2 and 5 on the 13th day and a significant change in these attributes for formulation 4 on the 25th day of storage.

Changes in the texture profile during the storage can be triggered by proteolysis, glycolysis, lipolysis, and pH changes. These alterations, in addition to the full solubility of the cheese constituents and the continuous enzymatic activities cause a change in the texture (Lucey, Johnson, & Horne, 2003). Furthermore, the increase in acidity during the storage may also cause changes in the characteristics of protein aggregates and in the texture profile (Queiroga et al., 2013).

In general, the different formulations showed no significant variations on the parameters evaluated during development and storage. Thus, is possible to designate the formulations with lower cream contents, once consumers seek products with healthier and more functional properties. Furthermore, a high cream content may not be desirable, as it has been associated with increased health risk (Andrade, Mattos, Carvalho, Machado, & de Oliveira, 2013).

Therefore, taking into account the desirable final characteristics of the product, especially acceptability by consumers, two products with reduced cream contents could be suggested for marketing: formulations 1 and 3 (high chia and quinoa content, respectively). The properties of these seeds suggest their use as functional ingredients with a high potential for application in this type of product by improving food quality and nutritional enrichment. In addition, products containing chia or quinoa represent new products that satisfy a new market demand. Furthermore, the addition of components that have human health benefits and present nutritional and technological advantages, such as increases in moisture retention, texture and elasticity, justify the use of chia and quinoa seeds as enrichments.

3.2.5. Microbiological analyses

The cheese formulations showed suitable sanitary conditions during storage, compatible with current Brazilian regulations (ANVISA, 2001; MAPA, 1996). *Staphylococcus auereus* was absent in

Table 4
Texture profile of cream cheeses developed.

Storage (Days)	Samples	Hardness (N)	Adhesiveness (N × s)	Cohesiveness	Gumminess (N)	Chewiness (N)	Resilience
1	1	0.15 ± 0.01^{dA}	0.37 ± 0.13^{dA}	0.82 ± 0.05^{aA}	0.13 ± 0.00^{aA}	0.13 ± 0.00^{dA}	0.07 ± 0.05^{aA}
	2	0.24 ± 0.01^{cA}	1.62 ± 0.10^{cA}	0.72 ± 0.02^{bA}	0.17 ± 0.01^{aA}	0.17 ± 0.01^{cA}	0.01 ± 0.00^{abA}
	3	0.35 ± 0.02^{aA}	4.38 ± 0.33^{aA}	0.73 ± 0.01^{bA}	0.41 ± 0.28^{aA}	0.26 ± 0.02^{aA}	0.01 ± 0.00^{bA}
	4	0.31 ± 0.00^{bA}	3.57 ± 0.22^{bAB}	0.74 ± 0.01^{bA}	0.23 ± 0.00^{aA}	0.23 ± 0.00^{bA}	0.01 ± 0.00^{bA}
	5	0.32 ± 0.01^{bA}	3.56 ± 0.36^{bA}	0.70 ± 0.02^{bA}	0.22 ± 0.00^{aA}	0.22 ± 0.00^{bA}	0.01 ± 0.00^{bA}
13	1	0.16 ± 0.01^{dA}	0.26 ± 0.16^{cA}	0.87 ± 0.05^{aA}	0.14 ± 0.00^{bB}	0.14 ± 0.01^{cB}	0.05 ± 0.04^{aA}
	2	0.28 ± 0.01^{cB}	2.33 ± 0.28^{bA}	0.80 ± 0.02^{bC}	0.22 ± 0.01^{cB}	0.22 ± 0.01^{bB}	0.01 ± 0.00^{bB}
	3	0.34 ± 0.02^{abA}	2.91 ± 0.52^{bA}	0.72 ± 0.01^{cA}	0.25 ± 0.01^{aA}	0.25 ± 0.01^{abA}	0.01 ± 0.00^{bA}
	4	0.33 ± 0.02^{bA}	3.17 ± 0.34^{bB}	0.75 ± 0.02^{bcA}	0.25 ± 0.01^{aA}	0.25 ± 0.01^{abA}	0.01 ± 0.00^{bB}
	5	0.38 ± 0.02^{aB}	4.26 ± 0.30^{aAB}	0.69 ± 0.01^{cA}	0.26 ± 0.01^{aB}	0.26 ± 0.01^{aB}	0.01 ± 0.00^{bAB}
25	1	0.17 ± 0.01^{cA}	0.15 ± 0.08^{cA}	0.84 ± 0.03^{aA}	0.14 ± 0.00^{cB}	0.14 ± 0.00^{cB}	0.04 ± 0.01^{aA}
	2	0.29 ± 0.01^{bB}	1.93 ± 0.49^{bA}	0.74 ± 0.04^{bAB}	0.21 ± 0.00^{bB}	0.21 ± 0.00^{bB}	0.01 ± 0.00^{bB}
	3	0.40 ± 0.01^{aB}	3.74 ± 0.88^{aA}	0.68 ± 0.02^{bcB}	0.27 ± 0.01^{aA}	0.27 ± 0.01^{aA}	0.01 ± 0.00^{bB}
	4	0.40 ± 0.03^{aB}	4.55 ± 0.61^{aC}	0.66 ± 0.01^{cB}	0.27 ± 0.01^{aB}	0.27 ± 0.01^{aB}	0.00 ± 0.00^{bC}
	5	0.40 ± 0.01^{aB}	4.74 ± 0.20^{aB}	0.69 ± 0.01^{bcA}	0.28 ± 0.01^{aB}	0.28 ± 0.01^{aB}	0.00 ± 0.00^{bA}

The same lowercase letters in the same column indicate no significant differences among the formulations for the same storage time ($P < 0.05$). The same uppercase letters in the same column indicate no significant differences among the storage times for the same formulation ($P < 0.05$).

the samples. The absence of *Salmonella* spp. and *L. monocytogenes* was found in 25 g. The results for total and fecal coliform were <0.3 MP N/g. Total counts of aerobic mesophilic were approximately 1.0×10^4 CFU/g, and yeasts and molds were approximately 3.6×10^3 CFU/g. Therefore, the preservatives and the manufacturing process were sufficient to ensure the satisfactory sanitary conditions and microbial stability during the storage.

4. Conclusion

A novel protease from *Bacillus* sp. P45 after purification showed a high coagulating activity and the ability to hydrolyze milk proteins. The enzyme was efficiently used in the development of cream cheese enriched with chia and quinoa flour. The technological parameters demonstrated that formulations were highly stable and viable. The product was very stable, presenting a high level of water retention and showed high fiber contents, suggesting that the product may be consumed as a fiber source with health benefits. Moreover, the results indicate the feasibility of the purified enzyme as an alternative coagulant for the development of innovative biotechnological processes, such as the development of new dairy products with functional ingredients.

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