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Original article

Exogenous enzymes in dairy technology: acidic proteases from processing discards of shrimp *Pleoticus muelleri* and their use as milk-clotting enzymes for cheese manufacture

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Summary Argentina is the seventh largest world producer of cheese. Owing to the profitability of this industry, more than a thousand of dairy companies and farms exclusively produce cheeses, and, thus, this activity utilises 42% of total raw milk production of the country. Due to the increase in cheese demand, and because calf rennet is relatively expensive, this has motivated the study of new sources of proteases due to the potential use in biotechnological processes. Previously, crustacean proteases have been considered as good rennet substitutes for milk coagulation during the cheese making process. The aim of this study was to evaluate and characterise acidic proteases obtained from *Pleoticus muelleri* discards and their proteolytic capability as well as their potential use as a milk-clotting agent. The activity of aspartic proteases and the satisfactory acceptation by the taste panel for cheese made with shrimp's enzymes suggest that *P. muelleri* proteins offer a high potential for use in dairy biotechnological processes with potential use as milk-clotting agents for cheese making.

Keywords Acidic proteinases, biotechnology, cheese, dairy technology, discards, shrimp.

Introduction

Over the last 10 years, as a result of large investments, Argentina has recorded the highest growth in net export of dairy products after the United States. However, in the past two decades, the number of dairy units shrank from about 50 000 to 11 000. Despite this reduction, dairying still ranks sixth in job generation and fourth for equitable income distribution (Iglesias, 2012). In addition, Argentina has a relatively stable market, where consumption of cheeses per capita is 12 kg per year. Thus, the government is developing a new Strategic Plan for Argentina's Dairy Chain 2020 focusing on small- and medium-scale dairying (Dugdill *et al*., 2013).

In cheese production, milk clotting by calf rennet is the procedure most commonly used (Dantas *et al*., 2016; Felicio *et al*., 2016). However, the low supply of calf rennet and the incidence of bovine spongiform encephalopathy are incentives for the search of alternative enzymes (Pontual *et al*., 2012). Natural milk-clotting enzymes extracted from plants and microbial organisms or recombinant chymosin have gained the attention of dairy technology (Sousa *et al*., 2001; Tavaria *et al*., 2001). Interest in isolating proteolytic enzymes from marine species is growing, especially as possible substitutes for milk-clotting enzymes but also for accelerating cheese ripening (Han & Sahidi, 1995; Tavares *et al*., 1997; Rossano *et al*., 2005, 2011).

In this study, we have analysed the enzymes extracted from the shrimp *Pleoticus muelleri* (Bate, 1888). This species is an important fishing resource of the Argentine Sea, which has a large distribution area in south-west Atlantic waters from 20°S, Espíritu Santo, Brazil, to 50°S, Santa Cruz, Argentina (Boschi, 1986). The Argentine shrimp is frequently captured as bycatch and generally discarded because it is not the target species. Bottom trawling is the most common fishing technique and produces large amounts of discard and incidental captures with a wide variety of species, where *P. muelleri* represents 24.6–29.3% of the captured taxa discarded (Bovcon *et al*., 2013).

Previously, digestive proteinases from the midgut gland of *P. muelleri* were characterised in relationship with moulting stages and compared the dietary effect

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under culture conditions (Fernández-Gimenez *et al*., 2001; Díaz *et al*., 2008; Velurtas *et al*., 2011). In a preceding work, we investigated the properties of digestive enzymes in discard of this species by evaluating operational parameters, activity and inhibition of proteinases (Pereira, 2016). The aim of this study was to evaluate the proteolytic capability of *Pleoticus muelleri* enzymes as milk-clotting agent in cheese technology.

Materials and methods

Biological materials

The specimens of *P. muelleri* were obtained from hake commercial fleet discards in the waters of San Jorge Gulf (Fishing season, May 2012). This gulf is located in central Patagonia, southern Argentina, between 44° and 47°S and between 66 and 71°W (Sylwan, 2001). After catching, the specimens were stored at −20 °C until used.

For the proteolytic enzyme studies, ten individuals were taken at random. Whole shrimp were homogenised in chilled distilled water (1:4 w/v) for 20 s each time, using a kitchen blender operating at low speed. The homogenates were centrifuged (Presvac EPF-12R, Argentina) for 30 min at 4 °C and 10 000 ***g***. The lipid layer was removed, and supernatants were used as the enzyme extract.

Biochemical properties

Soluble casein (Sigma C7078, St. Louis, MO, USA) was used as substrate for the assay of proteolytic activity of *P. muelleri's* extracts, and this activity was compared with the hydrolysis of casein by commercial rennet *Tres Coronas*®. Proteinase activity at pH 6.5 was assayed using 1% (w/v) casein as substrate in 50 mM buffer Tris–HCl pH8 and adjusted to pH 6.5 with hydrochloric acid. The enzyme extract (100 μL) was added to 1.1 mL of casein solution and kept at 30 °C for 10 min. Then, 8 mL of 5% (w/v) TCA was added and the mixture was allowed to stand for 30 min in cold bath. Subsequently, mixtures were centrifuged at 5000 ***g*** for 20 min and the absorbance of supernatants was measured at 280 nm (Kunitz, 1947). For blanks, TCA was added before the substrate. Assays were done in triplicate. Each test was performed in triplicate. Caseinolytic activity units were calculated according to the following equation:

$$\mathrm{Ucas} = \left(\frac{A\,280\,\mathrm{nm}}{t \times V}\right) \times \mathrm{Fd}$$

Caseinolytic activity units (Ucas) were defined as the variation in units of absorbance resulting in 1 mL of enzyme solution, due to the products of digestion of casein soluble by TCA (5% w/v), per minute, at 37 °C and pH 6.5.

Duration time (min) = t

Volume of the enzymatic extracts tested (mL) = V

Dilution factor of the enzyme extract = Fd

Enzyme extracts were incubated with 1 μg mL^{-1} Pepstatin A (Sigma P5318) in methanol, as inhibitor of aspartic proteinases. Solutions (10 μL) of Pepstatin A were separately mixed with the enzyme extracts (10 μL) and incubated for 60 min at 25 °C. The samples with inhibitor were assayed for activity at pH 6.5 as described above. Assays were run in triplicate. Control assays contained inhibitor solvent without the inhibitor. Activity in inhibition assays was reported as percentage, considering the activity measure in the absence of inhibitor as 100%.

Capability of shrimp proteinases to make cheese

The effect of different concentrations (10, 50 and 100 mM) of $CaCl_2$ and NaCl on the enzymatic activity was studied. Enzyme activity measurement was carried out according to the protocol detailed above, using casein as substrate. Assays were done in triplicate.

The caseinolytic activity was assessed during 4 weeks, and samples were taken every 2 weeks. Activities were determined according to the protocol explained in previous section. Each test was performed in triplicate. Caseinolytic activity units were calculated as well.

Milk-clotting activity was measured following the procedure described by Arima *et al*. (1970) and modified by Beka *et al*. (2014). The substrate was prepared by dissolving commercial bovine skimmed milk powder (Ilolay, Santa Fé, Argentina) in 100 mL of 10 mM L^{-1} $CaCl_2$ to a final concentration of 12% w/v, pH 6.5 according to the procedure described by IDF (1992). The substrate (6 mL) was pre-incubated for 5 min at 37 °C. The next step was put in six test tubes, 1 mL of this substrate; then, in three tubes, we added 0.2 mL of shrimp enzyme extracts and in the remaining three tubes, 0.2 mL commercial rennet *Tres Coronas*®. Test tubes were rotated by hand, and the time was measured between the moment when reagents were mixed and the initial appearance of visible signs of coagulation as discrete particles were discernible. The one milk-clotting unit (UCL) was defined as the amount of extract enzyme required for clotting of 100 mL milk in 40 min at 37 °C and was calculated as

$$\mathrm{UCL} = \frac{2400 \times V}{t \times v}$$

where V equals the volume of milk (mL), v the volume of coagulant (mL) and t the clotting time in seconds.

The well-established model of semi-soft type cheese was used to investigate whether the *P. muelleri* extracts

are suitable for cheese making. Cheeses were produced according to the method described by Shakeel-Ur-Rehman *et al*. (1998) using either commercial rennet or *P. muelleri* enzymes as coagulants. Each coagulant, previously diluted with water to 300 μL to have equal milk-clotting activity, was added to 200 mL milk. Semi-soft Quartirolo-type cheeses were manufactured in batches on the same day using each of the two coagulants. Efficacy of extracts in the manufacture of semi-soft Quartirolo-type mini-cheeses was determined by assessing their proteolytic ability over time in comparison with cheese made with commercial rennet. Cheeses were ripened at 4 °C during 2 weeks.

The cheeses were evaluated using a score card by a tasting panel that was composed of 25 panellists (60% women, 40% men, aged 20–65) were recruited at random from the University of Mar del Plata to take part in the consumer test, and the selection criterion was choose people accustomed to eating cheese.

The cheeses were uniformly prepared and presented to panellist in isolated booths. Crackers and water were used as neutralising agents between samples. The principal objective of this discriminative sensory test was to evaluate whether there were any significant differences in flavour, body, texture and colour between the cheese made with shrimp enzymes and with commercial coagulant (Balthazar *et al*., 2016). The tasting was blind, using coded samples, so tasters do not have knowledge of the origin of the coagulant milk. Then, an index card of cheese tasting was completed for each taster that was decoded and analysed by statistical procedures. Outward appearance (format and skin) and inner aspect (colour and the structure of the mass of cheese) were considered (Table 1). The texture in the mouth was tested, including several aspects such as texture on the palate, intensity and quality of the odour and flavour as well as persistence in mouth (Table 2).

Table 1 Index card: aspect (surface and form)

Score value	1	2	3	4	5	6	7	Coefficient	Partial Score	Total Score value
Outward appearance								2		
Inner aspect								3		

Score scale value: bad (1); deficient (2); regular (3); acceptable (4); good (5); very good (6); excellent (7). Partial score = score value × coefficient. Maximum total score value: 35.

Table 2 Index card: texture in mouth and smell-taste

Score value	1	2	3	4	5	6	7	Coefficient	Partial Score	Total Score
Texture on the palate								4		
Odour (scent)								3		
Intensity and quality of the odour and flavour								6		

Score scale value: bad (1); deficient (2); regular (3); acceptable (4); good (5); very good (6); excellent (7) Partial score = score value × coefficient. Maximum total score value: 105.

Statistical analysis

The differences in enzyme activity and the results of tasting panel were compared with ANOVA after testing data normality and homogeneity of variance. Significant differences were considered at $P \leq 0.05$. When significant differences were found, a Tukey–Kramer Multiple Comparison test was performed to locate these differences (Sokal & Rohlf, 1994). Analyses were made using NCSS 8 Software (NCSS LLC, Kaysville, UT, USA).

Results

The caseinolytic activity of *P. muelleri's* enzymes and commercial rennet *Tres Coronas*® were studied and compared among them. The results showed statistical differences between treatments: shrimp enzyme extract has a higher activity on casein substrate than commercial rennet, with values of 1.4 ± 0.04 and 0.9 ± 0.01 Ucas, respectively. To confirm the capability of aspartic proteinases in milk clotting, the caseinolytic activity of shrimp protein extract was evaluated with specific inhibitor, and the enzyme activity was inhibited 69.6% by Pepstatin A.

The effect of $CaCl_2$ and NaCl on enzyme activity with casein (pH 6.5) as substrate is shown in Table 3. The caseinolytic activity was increased significantly with 50 mM $CaCl_2$; however, when NaCl was added, the activity recorded was 4.88-fold, 2.63-fold and 2.22-fold higher than control with 10, 50 and 100 mM, respectively.

To evaluate the storage effect on caseinolytic activity, the enzymes extracts were kept at 4 °C for 4 weeks. Samples to assess such activity were taken every 2 weeks. The initial activity was 1.4 ± 0.04 Ucas and decreased around 10% at the second week (1.3 ± 0.04 Ucas). On the fourth week, the activity declined about 40% (0.6 ± 0.04 Ucas) (Fig. 1).

Milk-clotting activity was evidenced in the assays carried out using shrimp proteases as coagulant and also with the commercial rennet *Tres Coronas*®. The values registered were 0.34 ± 0.009 and 0.25 ± 0.008 UCL (milk-clotting unit), respectively, with results

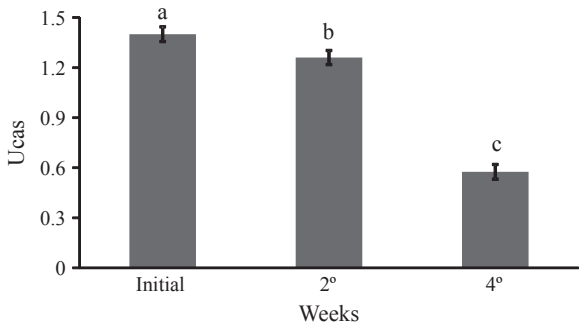

Figure 1 The effect of storage on the caseinolytic activity of *Pleoticus muelleri* enzymes, during 4 weeks. Different letters indicate significant differences between treatments ($P \leq 0.05$). Ucas: caseinolytic activity units.

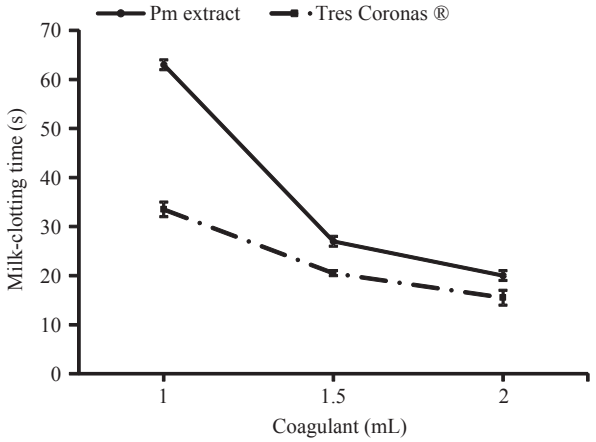

Figure 2 Variation of milk-clotting time with different amounts of enzymatic extract of *Pleoticus muelleri* and commercial rennet.

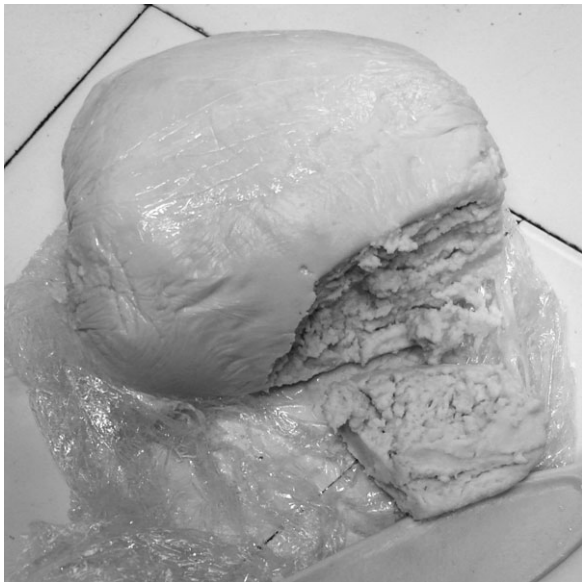

Figure 3 Quartirolo cheese made with shrimp protein extract. The image shows a cheese with firm and compact body.

Table 3 Caseinolytic activity of enzyme extracts of *Pleoticus muelleri* incubated with different concentrations of NaCl and $CaCl_2$

Caseinolytic activity			
NaCl		$CaCl_2$	
mM	Ucas	mM	Ucas
–	0.67 ± 0.352^{a}	–	0.67 ± 0.352^{a}
10	3.27 ± 0.484^{b}	10	0.66 ± 0.191^{a}
50	1.77 ± 0.221^{c}	50	3.17 ± 0.228^{b}
100	1.49 ± 0.583^{ac}	100	1.07 ± 0.609^{a}

Means with different lowercase superscript on the same column indicate presence of statistical difference ($P \leq 0.05$).

denoting differences among means. In addition, the clotting time was evaluated with different volumes of shrimp proteases and commercial rennet *Tres Coronas*®. The shrimp enzymes took twice as long as the commercial rennet when 1 mL was used but only a few seconds longer with 1.5 or 2 mL (Fig. 2).

The results of organoleptic assessment of cheese prepared with shrimp proteases and commercial rennet as coagulants are summarised in Table 4. The appearance parameters (surface and form) of cheeses were rated as satisfactory by the tasters. When the taste panels tried the cheese made with *P. muelleri* extracts, they considered that its texture in mouth and smell-taste were very acceptable, while these parameters were considered just as acceptable for the cheese made with commercial rennet; furthermore, the two kinds of cheese had firm and compact bodies (Fig. 3). In addition, for cheeses made with shrimp enzymes, off-flavour or bitterness was not noted by any member of the panel, while a slightly acidic flavour, good body and texture were noted in cheeses made with commercial rennet (Table 4).

Discussion

In the last years, the dairy sector of Argentina has increased the export of cheeses and, in consequence, sales have been incremented about 20%. Owing to the profitability of this industry, more than a thousand of dairy companies and farms exclusively produce cheeses, and thus, this activity utilises 42% of total raw milk production of the country (Ministerio de Agroindustria, 2016). The increased demand of cheese and the use of calf rennet, as conventional milk-clotting enzyme, is widely used in cheese making around the world. However, in many countries, the

Table 4 Results of tasting of cheeses. Organoleptic quality of cheeses made with both coagulants

Sensory characteristic	Maximum score (points)	Cheese made with	
		shrimp extract	commercial rennet
Aspect (surface and form)	35	33 ± 0.8[a]	30 ± 0.5[b]
Texture in mouth and smell-taste	105	104 ± 0.7[a]	96 ± 0.9[b]

Means of twenty-five tasters ± standard error. Means with different lowercase superscript on the same row indicate presence of statistical difference ($P \leq 0.05$).

cruel procedure to extract the calf rennet from the calf's fourth stomach, the short life and the high price of rennet have encouraged the search for substitutes such as the recombinant chymosin produced by bacteria and fungi (Sousa *et al*., 2001; Broome *et al*., 2006).

Recently some plant proteases have been considered as good alternatives for milk coagulation during the cheese making process (Reis *et al*., 2000; Pintado *et al*., 2001; Fernández-Salguero *et al*., 2002; Rocha *et al*., 2010; Mazorra-Manzano *et al*., 2013; Beka *et al*., 2014). Interestingly, only a few digestive enzymes from marine species (such as harp seal (*Pagophilus groenlandicus*), crayfish (*Pacifastacus astacus*), langostilla (*Pleuroncodes planipes*) and tuna fish (*Thunus obesus*) were considered for their chymosin-like characteristics as potential substitutes for rennet (Shamsuzzaman & Haard, 1983, 1984; García-Carreño *et al*., 1994; Tavares *et al*., 1997). Recently, Li *et al*. (2010) demonstrated that the marine yeast, *Metschnikowia reukaufii*, produces an acidic protease with milk-clotting activity; on other hand, Rossano *et al*. (2011) found a moderate clotting activity in the extracts of the *Munida* crustaceans. The authors of this study have been working with *P. muelleri*'s enzymes and demonstrated that this species has a high aspartic enzyme activity, term stability and activity in a wide range of pHs (Pereira, 2016). This context has motivated the screening and study of new sources of milk coagulant, by reusing shrimp enzymes obtaining from fisheries discards.

Aspartic proteases, also known as acidic proteases, are a subfamily of endopeptidases and are specifically inhibited by Pepstatin A. These enzymes are used as milk-coagulating enzymes for the manufacture of cheese and as additives to improve food flavour and texture (Nai-Wan *et al*., 2014). In the present work, on the basis of the effects of specific inhibitor, the enzymatic extract of *P. muelleri* registered a higher activity of aspartic proteases than *Munida* crustacean (D'Ambrosio *et al*., 2003; Rossano *et al*., 2011). Therefore, the protease extract of shrimp *P. muelleri* could be an appropriate milk coagulation enzyme for cheese manufacture.

Previous research has observed that milk-clotting activity from the protein extract of vegetal species as *Moringa oleifera* flowers, *S. dobium* and *Wthania* seeds, *Bromelia hieronymi* fruits and *Cynara scolymus* flowers is $CaCl_2$ dependent (Chazarra *et al*., 2007; Naz *et al*., 2009; Ahmed *et al*., 2010; Bruno *et al*., 2010). In the present study, we evaluated the enzymatic extract stability with 10, 50 and 100 mM of $CaCl_2$. As a result, we found that the addition of 50 mM increased the enzyme activity with casein as substrate, perhaps because this cation helps to destabilise casein micelles and then increase its hydrolysis. Clotting of milk is a result of the action of proteases that destabilise casein micelles. The caseins α and β are localised within the micelle, whose structure is maintained in solution by the κ casein hydrophilic domain. The hydrolysis of κ casein results in the collapse of micelle and exposure of α and β caseins to calcium, leading to separation of milk into a solid (clot or curd) and liquid (whey) phases. The $CaCl_2$ forms bridges between positive and negative charges on casein micelles, causing them to break and releasing α and β caseins. Curd is formed due to associations between these proteins and calcium (Pontual *et al*., 2012).

A significant variation of milk NaCl content in the central production area of Argentina was previously reported; furthermore, some studies have reported that milk coagulation is influenced by NaCl content (Sbodio & Revelli, 2012). For this reason, we evaluated the stability of caseinolytic activity at different NaCl concentrations (10, 50 and 100 mM) and it has demonstrated that activity was enhanced when NaCl was added. These results suggest that the NaCl concentration could allow the caseinolytic activity to enhance the cheese manufacturing process.

The enzymatic coagulation of milk is the modification of casein micelles via limited hydrolysis of casein by rennet, followed by calcium-induced micelle aggregation (Fox & McSweeney, 2004). Clotting time is usually defined as the time from enzyme addition until the observation of graininess or flecks in a revolving bottle, or test tube. A number of researchers have investigated the relationship between clotting time and objective parameters generated from instruments based on optical principles (Castillo *et al*., 2010). In this work, test tubes were periodically rotated by hand and time was measured between mixing the reagents and the initial appearance of visible signs of coagulation as discrete particles become discernible. According to D'Ambrosio *et al*. (2003), protein extract of *Munida* crustaceans used as alternative coagulant of milk registered a coagulant activity 150 times lower than

traditional liquid calf rennet (*Clerici*®) and 80 times lower than common lamb rennet pastes. Our results demonstrated that the rennet *Tres Coronas*® is 1 time faster when we used 1 mL of coagulant and 0.3 times faster when using volumes of 1.5 and 2 mL, allowing us to conclude that the milk-clotting time is dependent on the coagulant concentration. This interpretation is in accordance with previous studies, which assert that clotting time decreases when enzyme concentration is increased (Carlson *et al*., 1987; López *et al*., 1998).

Proteolytic enzymes have been used to modify food proteins in order to improve texture, flavour, nutritional quality and functionality (Dong *et al*., 2008). Shamsuzzaman & Haard (1985) made a cheese prepared with seal gastric protease and showed significantly higher sensory scores than cheese coagulated with calf rennet. Moreover, Cheddar-type cheeses have been manufactured using enzymes extracted from the marine crustacean *Munida*, which show a higher extent of degradation of β casein than cheeses made using chymosin as a coagulant (Rossano *et al*., 2005). The same authors made a miniature Pecorino-type cheese using the enzyme extracts from the midgut gland of *Munida* and demonstrated that the crustacean enzymes are capable of degrading caseins in an original manner, but no sensory-palatable test was conducted (Rossano *et al*., 2011). Many authors recommended the use of trained panel using quantitative descriptive analysis (Morais *et al*., 2014; Gaze *et al*., 2015; Pereira *et al*., 2016); in this context, in our study, we manufactured and evaluated the organoleptic characteristics by discriminative test of Quartirolo-type cheeses using shrimp enzymes and commercial rennet. The appearance parameters (surface and form) of these cheeses were rated as satisfactory by the tasters. When the taste panel tried the cheese made with *P. muelleri* extracts, they considered that its texture in mouth and smell-taste were very acceptable. Those cheeses made with shrimp extracts had a positive acceptance by the panel, suggesting this product could easily be incorporated into the market.

This study showed that the extracts obtained from *P. muelleri* contain proteolytic and milk-clotting activity making it a feasible clotting enzyme for cheese production. Investigations are in progress to assess whether extracts from this shrimp could be used in the industry as a commercial rennet substitute or for the development of new cheese products. Nowadays, the authors of this study are trying to prepare a standardised formulation of coagulant enzymes from *P. muelleri*. Future workers may wish to investigate the pattern of proteolysis (as detected by gel zymography, RP-HPLC and MALDI-ToF mass spectrometry) of miniature Quartirolo soft type cheeses made using *P. muelleri* enzymes as coagulant.

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Conflict of interest

The authors declare no conflict of interest.

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