Effect of thermoresistant protease of *Pseudomonas fluorescens* on rennet coagulation properties and proteolysis of milk

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ABSTRACT

This study aimed to investigate the effect of different activity levels of a thermoresistant protease, produced by *Pseudomonas fluorescens* (ATCC 17556), on the cheesemaking properties of milk and proteolysis levels. Sterilized reconstituted skim milk powder was inoculated with the bacteria, and after incubation, centrifuged to obtain a supernatant-containing protease. Raw milk was collected and inoculated to obtain a protease activity of 0.15, 0.60, and 1.5 U/L of milk (treatments P1, P4, and P10, respectively). One sample was not inoculated (control) and noninoculated supernatant was added to a fifth sample to be used as a negative control. Samples were stored at 4°C for 72 h. After 0, 48, and 72 h, the rennet coagulation properties and proteolysis levels were assessed. The protease produced was thermoresistant, as no significant differences were observed in the activity in the pasteurized (72°C for 15 s) and nonpasteurized supernatants. The chromatograms and electrophoretograms indicated that the protease preferably hydrolyzed κ-casein and β-casein, and levels of proteolysis increased with added protease activity over storage time. The hydrolysis of α_S-caseins and major whey proteins increased considerably in P10 milk samples. At 0 h, the increase in the level of protease activity decreased the rennet coagulation time (RCT, min) of the samples, possibly due to synergistic proteolysis of κ-casein into para-κ-casein. However, over prolonged storage, hydrolysis of β-casein and α_S-casein increased in P4 and P10 samples. The RCT of P4 samples increased over time and the coagulum became softer, whereas P10 samples did not coagulate after 48 h of storage. In contrast, the RCT of P1 samples decreased over time and a firmer coagulum was obtained, possibly due to a lower rate of hydrolysis of β-casein and α_S-casein. Increased levels of protease could result in further hydrolysis of caseins, affecting the processability of milk over storage time.

Key words: psychrotrophic bacteria, milk storage, rennet coagulation property, thermoresistant protease

INTRODUCTION

The storage of raw milk for extended periods at low temperatures (2 to 6°C) on-farm has a significant effect on milk microflora, which becomes mostly composed of gram-positive and gram-negative psychrotrophic bacteria (Lafarge et al., 2004). Increased levels of psychrotrophs in raw milk could negatively affect the manufacture of dairy products, as those bacteria have the ability to produce heat-resistant extracellular proteases and lipases. Those enzymes can survive thermal treatments, such as pasteurization (e.g., 72°C for 15 s) or even UHT processing (138°C for 2 s or 149°C for 10 s), decreasing yield and sensory quality of dairy products during storage (Cousin, 1982; López-Fandiño et al., 1993). Muir (1996) suggested that raw milk with a psychrotrophic count higher than 5×10^6 cfu/mL should be rejected for processing, due to the possible high levels of lipases and proteases. The most common psychrotrophs found in raw milk during cold storage belong to the genus *Pseudomonas* spp., which secrete most of the heat-resistant peptidases during milk cold storage (Ercolini et al., 2009; Baur et al., 2015).

During the late exponential or early stationary growth phase, *Pseudomonas* bacteria produce thermoresistant alkaline metalloproteases (**AprX**), generally at a bacterial count of 10^7 to 10^8 cfu/mL (Stoeckel et al., 2016). These metalloproteases preferably hydrolyze κ-CN, then β-CN, and then α_{S1}-CN, and their activities are optimal at a pH between 7 and 9, in a temperature range between 30 and 45°C (Decimo et al., 2014; Martins et al., 2015). Zhang et al. (2018) reported that increasing concentrations of AprX resulted in increasing hydrolysis, mainly of κ-CN, and gelation in UHT milk. Bagliniere et al. (2013) inoculated 0.2 mg/L of purified AprX into milk and observed increased proteolysis of caseins over storage and destabilization of the

UHT milk produced after 8 d of storage. In the case of cheese manufacture, the activity of bacterial proteases can affect the casein content, which may result in low yield, off-flavor (i.e., bitterness), and texture problems (Cousin, 1982; Fox et al., 2017). Mankai et al. (2012) observed a low DM content in Gouda cheeses produced from milk stored at 4°C for 48, 72, and 96 h and related this to the activity of psychrotrophic proteases leading to losses of protein in whey. Boulares et al. (2011) also associated decreased cheese yield with enzymes produced by gram-negative psychrotrophs. High levels of proteolysis can negatively affect cheese manufacture and sensory characteristics of the final product; however, in contrast, certain levels of proteolysis are necessary during cheese ripening. Proteolysis is essential in most cheese varieties, as it is responsible for textural changes (i.e., hardness, elasticity, cohesiveness) and development of flavor during cheese ripening (Fox et al., 2017).

Some studies have correlated psychrotrophic bacterial counts with proteolysis levels in milk, highlighting the negative effect levels higher than 10^6 cfu/mL can have on cheesemaking properties of milk (clotting time, curd yield) and cheese sensory characteristics (Leitner et al., 2008; Ricciardi et al., 2015). However, psychrotrophs can produce a range of thermoresistant enzymes, which can be active in different conditions (e.g., temperature, pH). Therefore, in addition to psychrotrophic counts,

further studies are necessary to understand the activity and effect of different levels of proteases produced by psychrotrophs on the quality of milk used for cheese production. Those investigations could aid in elucidating to what extent those proteases could have a positive or negative effect on cheese manufacture. Therefore, the objective of this study was to investigate the effect of different activity levels of a thermoresistant protease, produced by a *Pseudomonas* strain, on the cheesemaking properties and proteolysis of milk.

MATERIALS AND METHODS

Experimental Design

The experimental design of this study is shown in Figure 1. Fresh raw milk was collected during milking from the milk line, inoculated with different levels of protease (control, negative control, 0.15, 0.60, and 1.50 U/L), and stored at 4°C for 72 h. After 0, 48, and 72 h, rennet coagulation properties and proteolysis levels in the samples were assessed. After transfer of supernatant containing the protease (time 0 h), milk samples were immediately prepared (pasteurized and pH adjusted) and the rennet coagulation properties were assessed. A maximum of 30 min elapsed between inoculation and assessment of properties. The experiment was repeated in triplicate over a 3-wk period.
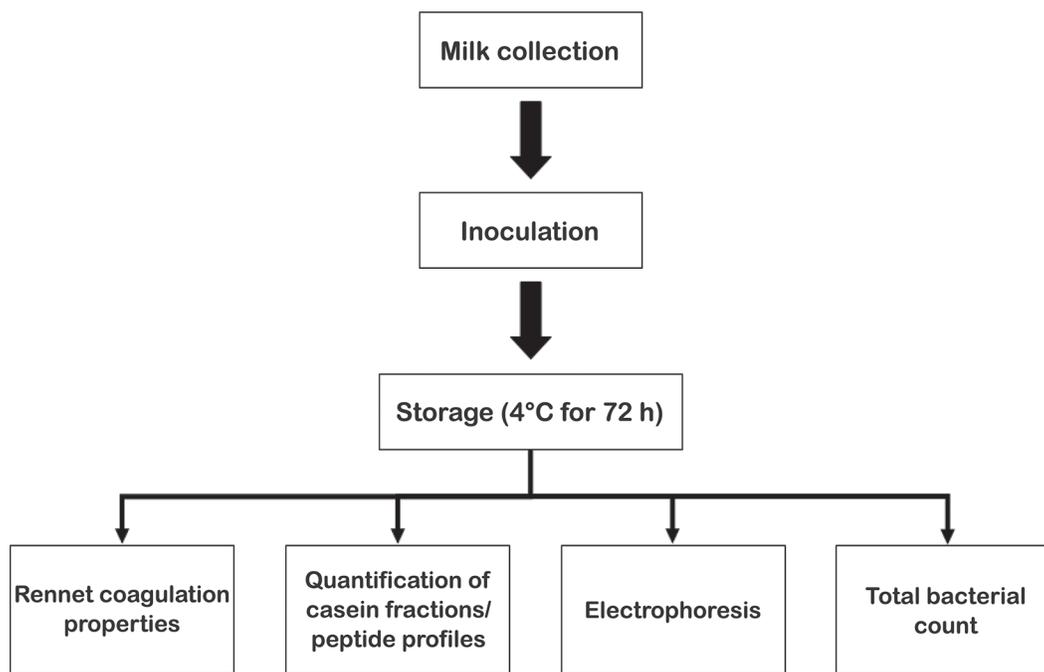
Figure 1. Experimental design: raw milk samples containing different levels of protease activity (0.15, 0.60, and 150 U/L) were stored at 4°C and rennet coagulation properties and proteolysis were assessed after 0, 48, and 72 h.

Culture of Pseudomonas fluorescens and Protease Extraction

Pseudomonas fluorescens (ATCC 17556) was grown in nutrient broth (meat extract 3 g/L, meat peptone 5 g/L; Sigma Aldrich, Dublin, Ireland) for 48 h at 26°C with stirring at 90 rpm. Afterward, 1 mL of the inoculated nutrient broth was spread-plated on blood agar plates (horse blood agar, 7% concentration, base No. 2, Oxoid, Basingstoke, UK) and plates were incubated for 24 h at 26°C. Bacterial colonies were transferred to 100 mL of sterilized reconstituted skim milk powder (**RSMP**; 10% wt/vol). The inoculated RSMP bottles were incubated for 120 h at 10°C with stirring at 90 rpm. Afterward, RSMP was centrifuged at 20,000 × *g* at ambient temperature for 30 min and the supernatant containing protease was collected.

Azocasein Assay

The production of protease by the strain was verified using azocasein (Sigma Aldrich, Dublin, Ireland) as substrate, as described by Andreani et al. (2016). The results were used to determine the activity of the protease. One unit of protease activity was defined as the required amount of enzyme able to hydrolyze azocasein to give an increase of 1 unit of absorbance per milliliter of sample (supernatant) per minute (Leighton et al., 1973). The following equation was used for calculations:

$$\text{Proteolytic activity} \left(\text{U} \cdot \text{mL}^{-1} \cdot \text{min}^{-1} \right) =$$

$$\frac{\left(\text{ABS}_{\text{sample}} - \text{ABS}_{\text{neg}} - \text{ABS}_{\text{blank}} \right) \times 10 \times F_d}{t},$$

where $\text{ABS}_{\text{sample}}$, ABS_{neg}, and $\text{ABS}_{\text{blank}}$ = absorbance of the sample, negative control, and blank, respectively; F_d = factor of dilution, which is equal to 1.00 as samples were not diluted; and t = time of reaction. In addition, samples of the same supernatant were separated in 2 groups to determine if the protease would still be active; after pasteurization, 1 group of 3 samples was heated to 72°C for 15 s, whereas the other 3 samples were not pasteurized. Afterward, those samples were analyzed using the azocasein assay for comparison.

Milk Collection and Inoculation

At the Teagasc Animal and Grassland Research and Innovation Centre (Moorepark, Cork, Ireland), spring-calving cows were milked in a 30-unit side-by-side milking parlor, with milking commencing at 0700 h. The milking equipment used, udder preparation, and sanitation of equipment were as described by Palu-

detti et al. (2018). Over 3 wk, raw milk was collected once weekly and transferred directly from the milking machine milk line to 5 × 500 mL sterilized bottles. Milk was transported to the Milk Quality Laboratory (Teagasc, Moorepark, Cork, Ireland) in cooling boxes.

Milk samples were inoculated based on the protease activity determined using the azocasein assay (0.030 ± 0.006 U/mL·min). The temperature of the samples was approximately 10°C at inoculation. Samples were inoculated with 5, 20, and 50 mL of supernatant containing protease per L of milk, corresponding to 0.15, 0.60, and 1.50 U/L of milk (treatments **P1**, **P4**, and **P10**, respectively). One milk sample was not inoculated to be used as control (**CON**), whereas 1 sample of milk was inoculated with 50 mL of supernatant from RSMP that was not inoculated with *P. fluorescens* and was used as the negative control sample (**P10 neg**). All milk samples were stored at 4°C for up to 72 h.

Total Bacterial Count

After 0, 48, and 72 h, milk samples from each treatment were tested in duplicate for total bacterial count (**TBC**). The analysis was performed according to the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004). The TBC was estimated using Petrifilm aerobic count plates, a ready-to-use medium (3M, Technopath, Tipperary, Ireland). Samples were incubated for 48 h at 32°C (Laird et al., 2004).

Rennet Coagulation Properties

The rennet coagulation properties of the inoculated milk were assessed using a Formagraph instrument (model 11700, Foss Electric, Hillerød, Denmark) after 0, 48, and 72 h of storage. Before analysis, milk samples were treated in a manner that mimicked the procedures applied during the manufacture of Cheddar cheese. Therefore, before adding rennet, samples were subjected to a heat treatment, mimicking industrial processing conditions. After inoculation, samples were placed in a water bath at 72°C and temperature was monitored using a thermometer. Samples were maintained in the water bath for 15 s after their temperature reached 72°C (approximately 5 min). Immediately after this time, samples were transferred to a water bath at 32°C, which is the usual temperature of milk at the start of Cheddar cheese manufacture. The pH of the samples was measured and, when necessary, adjusted to 6.55 with 4% (vol/vol) lactic acid solution. The milk volumes were renneted with chymosin (1:20 vol/vol dilution of double-strength Chy-max, Pfizer Inc., Milwaukee, WI) at a rate of 36 µL/10 mL, incubated at 32°C, and the coagulation properties monitored over a 30-min period.
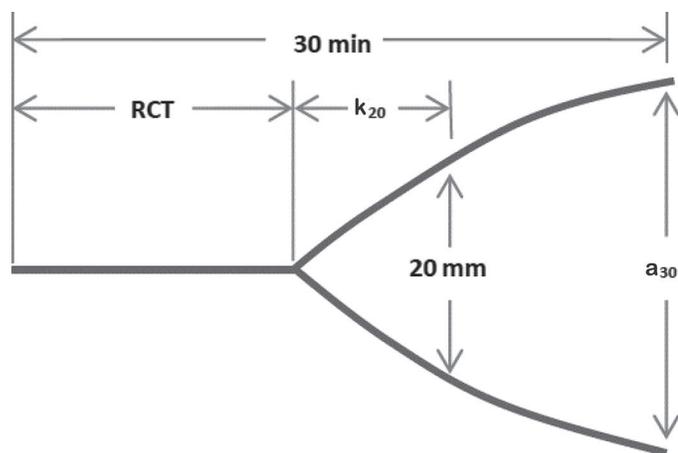
Figure 2. Schematic presentation of milk coagulation properties [rennet coagulation time (RCT), curd-firming time (k_{20}), and curd firmness at 30 min (a_{30})] measured by lactodynamography (McMahon and Brown, 1982).

The properties measured were rennet coagulation time (**RCT**, min), curd firmness at 30 min (**a_{30}**, mm), and curd-firming time (**k_{20}**, min). Rennet coagulation time is the time from the addition of rennet until the viscosity of milk starts changing (coagulation), k_{20} is the time from end point of RCT until a curd firmness of 20 mm is reached, and a_{30} is the firmness of curd in mm reached 30 min after the addition of rennet (McMahon and Brown, 1982; Figure 2).

Quantification of Casein Fractions

The casein fractions (κ-CN, α_{S1}-CN, α_{S2}-CN, β-CN, α-LA, β-LG A, β-LG B, and total casein) were identified and quantified (in duplicate) in the inoculated milk samples by HPLC. Milk samples (200 μL) were diluted in 3,780 μL of dissociating buffer (7 M urea and 20 mM Bis-Tris propane, pH 7.5). Twenty microliters of mercaptoethanol were added to the diluted samples, after which they were filtered through 0.22-μm filters. The equipment used was an Agilent 1200s system (Agilent Technologies, Santa Clara, CA) with a quaternary pump and a multiwavelength detector. The casein fractions were separated in the reversed-phase mode using an Agilent Poroshell 300SB C18 column (2.1 mm × 7.5 mm; Agilent Technologies). The gradient elution and peak detection were performed according to the method of Mounsey and O'Kennedy (2009).

Peptide Profiles

The peptide profiles of milk samples collected after 0, 48, and 72 h were obtained by HPLC. The nonprotein fractions of the milk samples were extracted using tri-

chloroacetic acid, according to the procedure described in IDF method 20–4 (IDF, 2001). The extracts were filtered using 0.45-μm syringe cellulose filters (25 mm diameter, Chromafil Xtra RC-45/25, Macherey-Nagel, Dublin, Ireland) and 50 μL were injected onto the column to obtain the profiles. The separation of milk peptides was performed in the reversed-phase mode using an Agilent Zorbax 300SB C8 column (4.6 mm i.d. × 150 mm, Agilent Technologies). The gradient elution and peak detection methodology was an adaption of the methodology of Rohm et al. (1996). The HPLC equipment used was an Agilent 1200s system (Agilent Technologies) with a quaternary pump and a multiwavelength detector.

SDS-PAGE

The individual proteins of the milk samples collected after 0, 48, and 72 h of storage were identified using pre-cast SDS-PAGE (Novex Technologies, Thermo Fisher Scientific, Waltham, MA) under reducing and nonreducing conditions. The inoculated milk samples were diluted in Milli-Q water to a protein concentration of approximately 5.7 μg/μL, and then further diluted in SDS sample buffer [NuPAGE LDS Sample Buffer (4×), Thermo Fisher Scientific)]. For reducing SDS-PAGE conditions, dithiothreitol [NuPAGE Sample Reducing Agent (10×); concentration = 500 mmol/L (Thermo Fisher Scientific)] was added to the samples at a level of 10% (vol/vol) of the sample total volume. Samples were heated to 70°C for 10 min, cooled, and loaded on 12% Bis-Tris SDS-PAGE gels (10 μg/well). A low-range protein ladder was also loaded on the gels (10 μg/well; Spectra Multicolor Low Range Protein Ladder, Thermo Fisher Scientific). The gels were run in SDS running buffer [NuPAGE MOPS SDS Running Buffer (1×)] at 200 V for 50 min. After, the gels were stained for 24 h with Instant Blue Coomassie (Expedeon, Cambridge, UK).

Statistical Analysis

The results of the azocasein test for the pasteurized and nonpasteurized supernatants were statistically compared using the MIXED procedure (Student's t-test) in SAS 9.3 (SAS Institute Inc., Cary, NC).

Least squares means for the main effects of storage time and added protease activity, as well as the interaction between storage time and added protease activity, were calculated using the GLIMMIX procedure also in SAS 9.3. A repeated-measures ANOVA was used, where storage time is a within-subject factor and level of protease activity is a between-subject factor. The fixed effects included in each model were storage time (0, 48,

Table 1. Mean (±SE) total bacterial count (TBC) of control (CON) and negative control (P10 neg) milk samples, as well as samples inoculated with 0.15, 0.60, and 1.50 U/L protease (treatments P1, P4, and P10, respectively), stored for up to 72 h

TBC ($\log_{10}$ cfu/mL)	Protease activity					
	CON	P10 neg	P1	P4	P10	SE
0 h	3.11^A	3.22^A	4.14^A	4.46^A	4.77^A	0.24
48 h	3.96^{AB}	4.26^A	5.45^A	6.00^B	6.00^B	0.24
72 h	5.33^B	5.30^B	8.00^B	8.00^C	8.00^C	0.24

$^{A–C}$Values within a column not sharing common superscripts differ significantly ($P < 0.05$).

and 72 h) and added protease activity (CON, P10 neg, P1, P4, and P10). The inoculated milk volumes within trial week were considered the experimental unit. The response variables were pH; TBC; RCT, k_{20}, and a_{30}; and κ-CN, α_{S1}-CN, α_{S2}-CN, β-CN, α-LA, β-LG A, β-LG B, and total casein. Residual checks were made to ensure that the assumptions of the analysis were met. The Tukey test (at 5% error probability) was used to compare the means for all variables.

RESULTS

Total Bacterial Count

The TBC of milk samples from different treatments and stored for up to 72 h are shown in Table 1. The TBC of the samples significantly increased with level of protease activity ($P < 0.0001$) in the milk samples and over storage time ($P < 0.0001$; Table 1). The interaction between storage time and added protease activity did not affect TBC ($P > 0.05$).

Measurements of pH in Milk

Protease activity did not affect the pH of the samples ($P = 0.52$). The mean (±SE) pH measured in CON, P10 neg, P1, P4, and P10 samples were 6.51, 6.53, 6.53, 6.54, and 6.53 ± 0.01, respectively. Storage time also did not affect pH ($P < 0.0001$), nor did the interaction between added protease activity and storage time ($P = 0.01$). We did not observe decreases in pH over storage time, except in P10 samples. At 0, 48, and 72 h the pH measured in P10 samples were 6.58, 6.55, and 6.47 at 0, 48, and 72 h, respectively ($P = 0.001$).

In addition, the mean (±SE) fat (3.44 ± 0.30%) and protein (3.58 ± 0.06%) contents did not differ between treatments and during storage of milk ($P > 0.05$; data not shown).

Azocasein Assay and Protease Activity

The mean (±SE) result obtained for the supernatant samples that were not pasteurized was 0.547 ± 0.109

nm. Based on this results, the mean (±SE) protease activity calculated in the supernatant was 0.030 ± 0.006 U/mL·min. Also, the result obtained for nonpasteurized samples did not differ from the results obtained for the samples that were pasteurized (0.535 ± 0.114 nm; $P > 0.05$).

Proteolysis

Casein Fractions. The significance of the main effects of storage time, added protease activity, and interaction between added protease activity and storage time on the concentration of caseins and major whey proteins of milk are shown in Table 2. The concentrations of those proteins measured in the milk samples from the different treatments stored for up to 72 h are also shown in Table 2.

Storage time affected the concentrations of α_{S2}-CN and β-CN. Protease activity and interaction between protease activity and storage time affected the concentrations of all caseins (Table 2).

Storage time affected the mean concentrations of α-LA, added protease activity affected the mean concentrations of β-LG A and the interaction between those factors affected mean concentrations of β-LG B.

The casein chromatograms of the CON, P10 neg, P1, P4, and P10 samples, stored for up to 72 h, are shown in Figure 3. The chromatograms of CON and P10 neg samples are similar and indicated low levels of proteolysis over storage. In relation to the inoculated samples, the levels of proteolysis increased with the activity of protease over storage time. The chromatograms indicated that κ-CN and β-CN were the main proteins hydrolyzed by the protease over time. We also observed small decreases in α_{S1}-CN peaks with increasing added protease activity. In P10 samples, decreases in the concentrations of α_{S1}-CN and α_{S2}-CN after 48 h and decreases in the concentrations of β-LG A and B after 72 h were greater compared with other samples (Table 2).

Peptide Profiles. The peptide profiles of CON, P10 neg, P1, P4, and P10 samples, stored for up to 72 h

at 4°C, are shown in Figure 4. The peptide profiles of the CON and P10 neg samples were similar and low levels of proteolysis were observed. Over storage time, the hydrolysis of proteins in milk increased with added protease activity.

SDS-PAGE. Nonreducing and reducing SDS-PAGE electrophoretograms of all milk samples stored for up to 72 h are shown in Figures 5A and 5B, respectively.

The electrophoretograms of the CON (lane 1) and P10 neg (lane 5) samples were similar and no apparent indication of proteolysis was observed. In contrast, the electrophoretograms of P1, P4, and P10 samples indicated that the protease was active mainly on κ-CN and β-CN, bands for which became less intense and decreased in size over time. In addition, we noted a small decrease in bands corresponding to α_{S1}-CN with increasing level of protease activity. The decrease was more noticeable in P10 samples.

The intensity of the peptide bands from 10 to 25 kDa, which are products of the hydrolysis of caseins, increased with level of protease activity. We observed those bands in samples tested after inoculation (0 h of storage), indicating that the protease is highly active in milk. Also, a peptide band around 14 kDa (above α-LA) was observed in lanes corresponding to P1, P4, and P10 samples (lanes 2, 3, and 4, respectively) and its intensity increased with level of protease activity and storage time.

Rennet Coagulation Properties. The significance of the main effects of storage time, added protease activity, and interaction between added protease activity and storage time on the rennet coagulation properties of milk are shown in Table 3. The values for RCT, k_{20}, and a_{30} obtained for the milk samples from the different treatments, stored for up to 72 h, are shown in Table 4. It was not possible to assess the rennet coagulation properties of the P10 milk samples after 48 h, as those samples did not form a coagulum after rennet addition.

Rennet coagulation time decreased significantly with increasing levels of protease activity (CON, P10 neg, P1, and P4: 21.54, 19.63, 16.64, and 16.81 ± 1.16 min, respectively; $P < 0.05$). Over 72 h, we observed a decrease in RCT when testing P1 samples. The results also indicated that RCT may tend to increase over time for P4 samples.

The interaction between level of protease activity and storage time affected a_{30} (mm) ($P = 0.002$). Over 72 h, a_{30} did not vary significantly for CON and P10 neg samples (Table 4). The variations in a_{30} measured in P1 samples were also not significant over 72 h; however, we observed an increase in that value after 48 h of storage. In relation to P4 samples, a_{30} decreased significantly after 48 h ($P = 0.001$; Table 4).

It was not possible to measure k_{20} value in P4 and P10 samples after 72 and 48 h, respectively, as those samples did not reach a curd firmness of 20 mm. Consequently, the data set obtained at 0 and 48 h was separated from the data obtained after 72 h for statistical analysis. After 48 h, the k_{20} measured for CON, P10 neg, P1, and P4 samples was not affected by storage

Table 2. Mean (±SE) contents of casein fractions (mg/mL) in control (CON) and negative control (P10 neg) milk samples and samples inoculated with 0.15, 0.60, and 1.50 U/L (treatments P1, P4, and P10, respectively), stored for up to 72 h, and significance of the main effects of protease activity, storage time, and the interaction between protease activity and storage time on those contents

Protease activity	Time (h)	Casein fraction (mg/mL)							
		κ-CN	α_{S1}-CN	α_{S2}-CN	β-CN	α-LA	β-LG A	β-LG B	Total casein
CON	0	5.30	13.53	3.01	12.71	1.13	1.96	2.62	39.75
	48	5.66	12.44	3.25	11.51	1.13	1.95	2.54	38.48
	72	6.32	13.36	3.54	12.37	1.18	2.10	2.60	41.46
P10 neg	0	6.20	14.42	3.93	13.18	1.26	2.20	2.67	43.88
	48	6.35	13.65	3.97	12.43	1.21	2.21	2.51	42.33
	72	5.95	13.60	3.73	12.38	1.21	2.03	2.42	41.33
P1	0	4.13	12.60	2.94	11.56	1.05	1.80	1.96	36.54
	48	4.96	14.79	3.72	11.25	1.23	2.11	2.74	40.83
	72	5.16	14.35	4.07	10.53	1.26	2.07	2.74	40.18
P4	0	4.04	13.14	3.47	11.63	1.11	1.96	2.69	38.05
	48	3.69	11.78	3.28	8.28	1.12	1.81	2.53	32.51
	72	4.13	13.54	3.85	8.64	1.27	1.76	2.85	36.02
P10	0	4.25	13.39	3.79	13.18	1.04	1.87	2.64	37.77
	48	3.70	8.05	2.89	7.72	1.04	1.87	2.40	27.68
	72	3.32	8.13	2.86	6.60	1.19	1.17	2.02	26.30
SE		0.25	0.31	0.14	0.28	0.05	0.08	0.15	1.05
Significance									
Storage time		0.40	<0.0001	0.09	<0.0001	0.02	0.14	0.47	0.01
Protease activity		0.002	<0.0001	0.01	<0.0001	0.22	0.02	0.06	0.0004
Protease activity × storage time		0.02	<0.0001	0.001	0.001	0.20	0.18	0.04	0.001
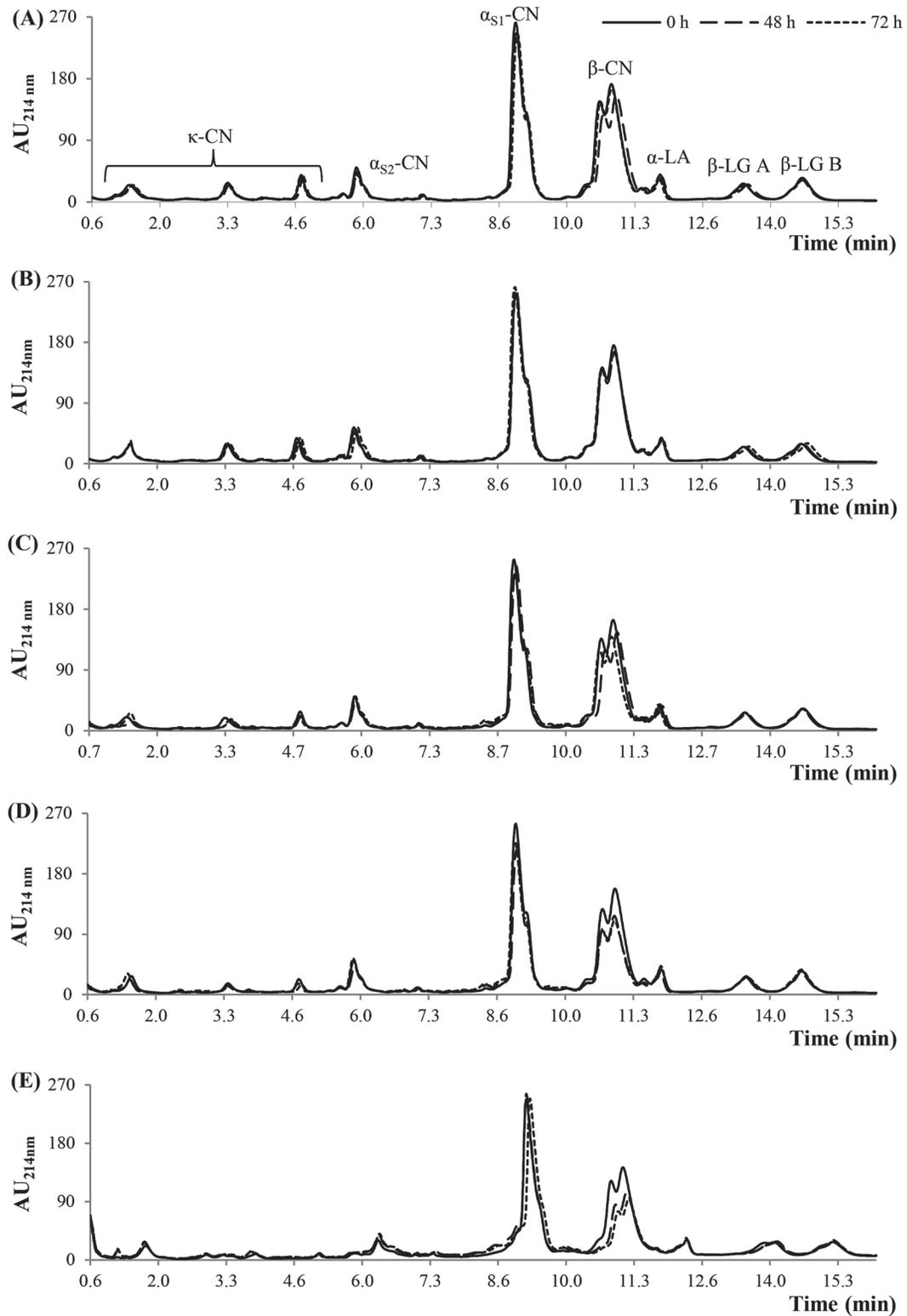
Figure 3. Separation of bovine milk caseins by reversed-phase HPLC (measured in absorbance units, AU). Chromatograms of (A) control and (B) negative control samples and samples inoculated with (C) 0.15, (D) 0.60, and (E) 1.50 U/L stored for up to 72 h are shown.
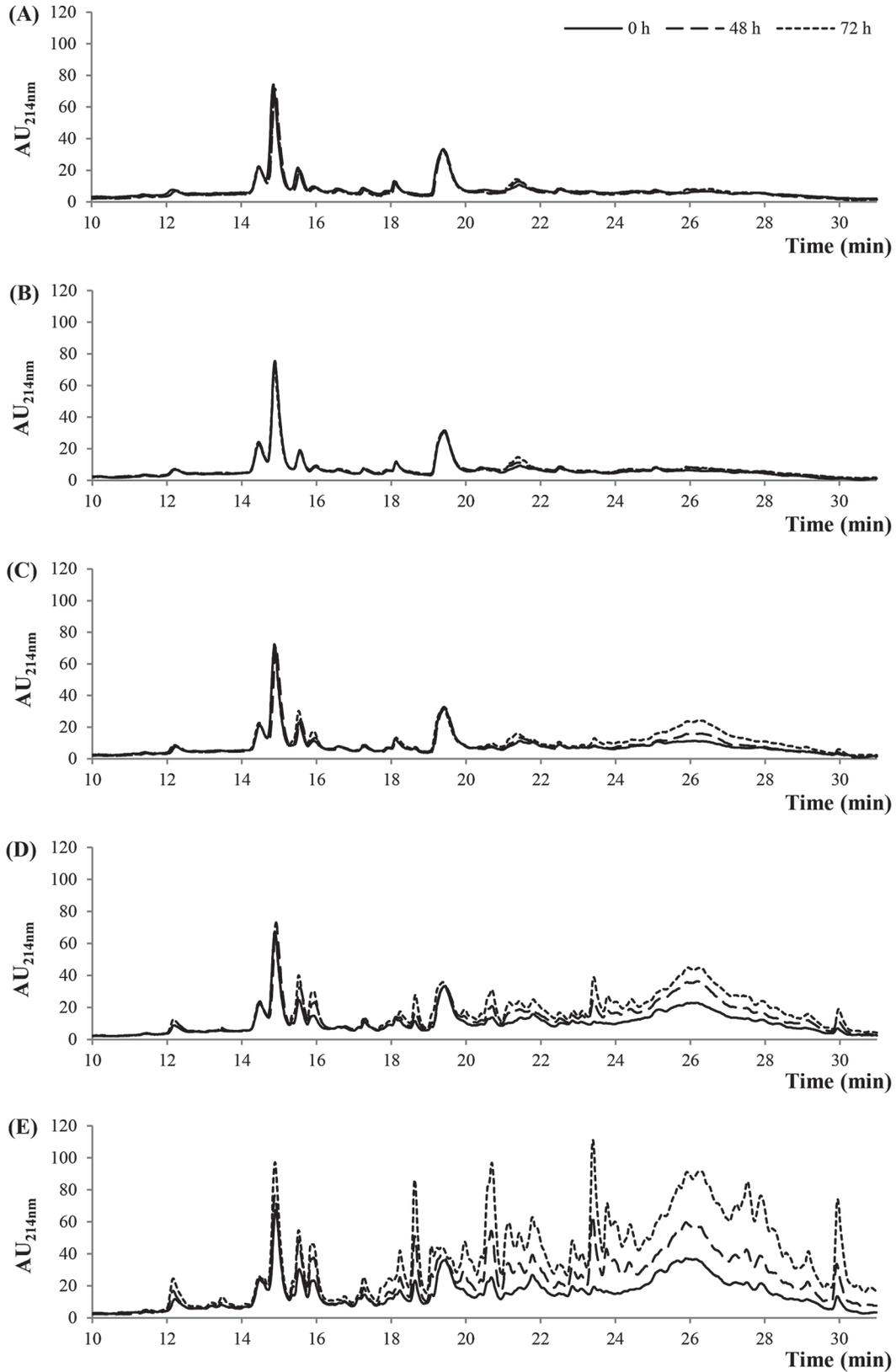
Figure 4. Separation of bovine milk peptides by reversed-phase HPLC (measured in absorbance units, AU). Chromatograms of (A) control and (B) negative control samples and samples inoculated with (C) 0.15, (D) 0.60, and (E) 1.50 U/L stored for up to 72 h are shown.

time ($P = 0.56$), added protease activity ($P = 0.32$), or interaction between those effects ($P = 0.09$). The CON, P10 neg, and P1 samples reached a curd firmness of 20 mm after 72 h, and the k_{20} for those samples did not differ ($P = 0.84$).

DISCUSSION

According to the azocasein test, the strain of *P. fluorescens* (ATCC 17556) produced active proteases when incubated at 10°C. This result is in accordance with
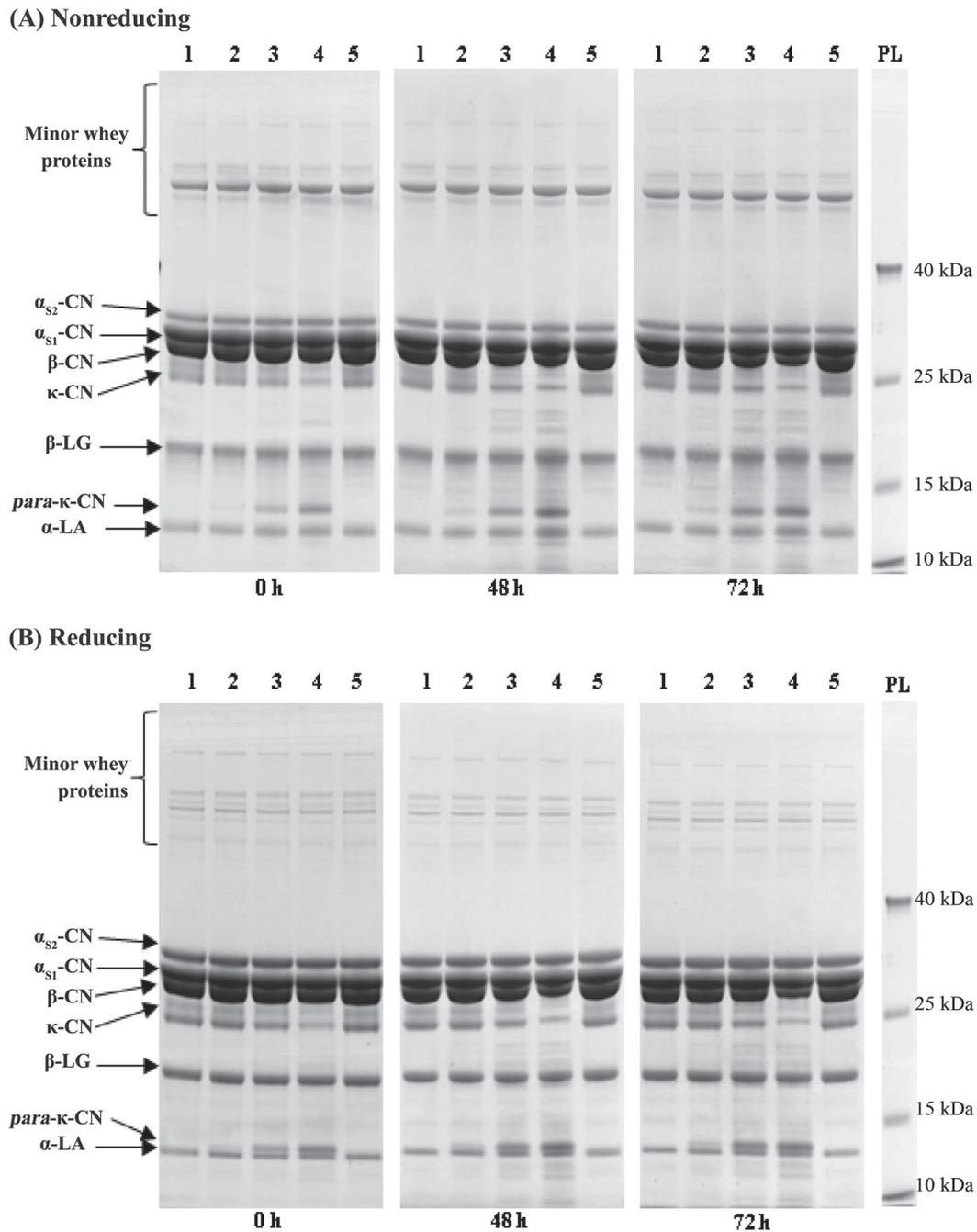
Figure 5. Sodium dodecyl sulfate-PAGE electrophoretogram of milk samples stored for up to 72 h under (A) nonreducing and (B) reducing conditions. Lane 1 corresponds to the control samples, and lanes 2 to 5 correspond to samples inoculated with 0.15, 0.60, and 1.50 U/L, and negative control, respectively. Lane PL corresponds to a low-range protein ladder.

the study from Marchand et al. (2009), in which the same strain presented proteolytic activity at 7, 22, and 30°C. In relation to the level of protease activity, Liao and McCallus (1998) cultured *P. fluorescens* CY091, centrifuged the medium, and obtained a protease activity varying from 5 to 8 U/mL in the supernatant. Those results differ from the activity obtained in this study, indicating that other strains may produce proteases with higher levels of activity. The production of protease could depend on several factors, such as bacterial strain, culture media, time, and temperature of incubation (McKellar, 1982).

The protease produced was thermoresistant, as its activity was maintained after pasteurization and therefore it would remain active during cheese manufacture. In addition, it is now widely shown that multiple *Pseudomonas* strains produce heat-resistant proteases (Bagliniere et al., 2013; Machado et al., 2017; Marchand et al., 2017).

The difference in TBC and bacterial growth rate between noninoculated samples (CON and P10 neg) and inoculated samples (P1, P4, and P10) over storage time at 4°C could be due to the presence of *P. fluorescens* cells in the supernatant used for inoculation. As the amount of supernatant added increased according to the aimed protease activity, the levels of TBC and bacterial growth rate also increased. The strains of *P. fluorescens* probably contributed to the increase in TBC because this strain can grow at temperatures up to 30°C, which is close to the incubation temperature applied to measure mesophilic bacterial numbers (32°C; Marchand et al., 2009).

After 72 h, the pH of P10 samples decreased significantly, which could be due to the high levels of bacteria in those samples. The high bacterial activity could have increased the acidity of milk with the conversion of lactose into lactic acid. The decrease in pH could have potentially affected the structure of the coagulum of P10 milk samples after 48 h, as the acidity could have

Table 3. The significance of the main effects of protease activity, storage time, and the interaction between protease activity and storage time on the rennet coagulation time (RCT, min) and curd firmness at 30 min (a_{30}, mm)

Rennet coagulation properties[1]	Significance		
	Storage time	Protease activity	Protease activity × storage time
RCT	0.06	0.02	0.21
a_{30}	0.06	0.08	0.002

[1]The significance levels obtained for k_{20} measurements were not included in this table as those measurements were separated in 2 groups (group 1: data obtained at 0 and 48 h; group 2: data obtained only at 72 h) for the statistical analysis, differently than for other measurements.

Table 4. Mean (±SD) rennet coagulation time (RCT, min), curd firmness at 30 min (a_{30}, mm) and curd-firming time (k_{20}, min) of control (CON) and negative control (P10 neg) milk samples and samples inoculated with 0.15, 0.60, and 1.50 U/L (treatments P1, P4, and P10, respectively), stored for up 72 h

Protease activity	RCT (min)			a_{30} (mm)			k_{20} (min)		
	0 h	48 h	72 h	0 h	48 h	72 h	0 h	48 h	72 h
CON[1]	23.0 ± 1.6^a	19.6 ± 0.9^a	22.0 ± 3.5^a	$17.36 \pm 2.3^{A,ab}$	$31.42 \pm 4.0^{A,a}$	$21.67 \pm 10.4^{A,a}$		5.39 ± 0.5^a	7.00 ± 0.8^a
P10 neg	17.7 ± 3.9^{ab}	19.5 ± 0.4^a	21.7 ± 2.5^a	$28.14 \pm 5.7^{A,a}$	$27.22 \pm 5.0^{A,ab}$	$25.10 \pm 10.1^{A,a}$		6.89 ± 1.8^a	6.39 ± 1.7^a
P1	20.3 ± 2.2^a	15.2 ± 1.1^a	14.4 ± 1.2^a	$23.27 \pm 6.1^{A,ab}$	$34.08 \pm 5.4^{A,a}$	$32.60 \pm 6.7^{A,a}$	6.58 ± 1.3^a	6.28 ± 1.3^a	7.28 ± 2.3^a
P4[1]	14.4 ± 6.5^{ab}	16.7 ± 3.9^a	19.3 ± 1.2^a	$32.78 \pm 9.0^{A,a}$	$17.93 \pm 6.5^{AB,b}$	$5.61 \pm 1.6^{B,b}$	7.86 ± 3.0^a	6.28 ± 1.3^a	
P10[2]	7.8 ± 4.0^b			27.98 ± 14.2^a			6.03 ± 2.2^a	9.31 ± 1.6^a	
							6.08 ± 0.6^a		

[A,B]Values within a row not sharing common superscripts differ ($P < 0.05$).

[a,b]Values within a column not sharing common superscripts differ ($P < 0.05$).

[1]Control samples and P4 samples did not reach a curd firmness of 20 mm after 0 and 72 h, respectively.

[2]Data missing after 48 and 72 h because the P10 sample did not coagulate.

influenced the net charge and interaction of the casein micelles (Fox et al., 2017).

The similarity between the results obtained for CON and P10 neg samples, as well as how they differed from the results obtained for the inoculated samples, indicated that the methods of production and extraction of proteases were effective and that the protease was active in milk.

The casein concentrations (Table 2), as well as the results from the chromatograms (Figures 3 and 4) and electrophoretograms (Figure 5), indicated an increase in proteolysis levels with added protease activity over storage time. The protease tested preferably hydrolyzed κ-CN and β-CN, whereas the rate of hydrolysis of α_S-CN increased with level of protease activity. The breakdown of α_S-CN and major whey proteins was more noticeable when the level of protease activity was increased to 1.50 U/L (treatment P10, Figures 3 and 5; Table 2). Similar results were observed by Zhang et al. (2015) when investigating the degradation on milk proteins by a thermoresistant protease produced by *P. fluorescens* BJ-10.

Statistical analysis indicated that α-LA and β-LG B levels were affected by storage time and interaction between added protease activity and storage time, respectively. However, we observed no trends over time for the concentrations on those proteins and variations were minimal (Table 2). We also observed small variations in the concentrations of whey proteins in the chromatograms (Figure 3). The only trend we observed was the decrease in the whey protein peaks when the protease activity was 1.50 U/L (treatment P10).

The hydrolysis of the caseins affected the rennet coagulation properties of the milk from different treatments. Overall, the increase in the level of protease activity resulted in a decrease in RCT. At 0 h, we observed that trend, which could be linked to the hydrolysis of κ-CN, an important casein for the coagulation of milk during cheesemaking. During coagulation, the Phe_{105}-Met_{106} bond of κ-CN is hydrolyzed and the N-terminal that remains bound in the casein network is called para-κ-CN. Due to changes in charges, the para-CN micelles are then able to aggregate in the presence of calcium ions (Fox et al., 2017). In the electrophoretograms (Figure 5), we observed a peptide band around 14 kDa (above α-LA) in lanes corresponding to P1, P4, and P10 samples (2, 3, and 4, respectively) and its intensity increased with level of added protease activity and storage time. Timotijevic et al. (2006) obtained a similar result when testing different concentrations of rennet, and the authors identified that band as corresponding to para-κ-CN. According to Jackman et al. (1985), psychrotrophic bacterial proteases include endopeptidases capable of hydrolyzing the phenylalanine-methionine

bond of κ-CN in a similar manner to rennet. Therefore, the increase in added protease activity could have resulted in increasing hydrolysis rates of κ-CN into para-κ-CN, and consequently decreased RCT in inoculated milk samples.

After 48 h, the RCT of P1 samples continued to decrease over storage time. In contrast, the RCT of P4 samples tended to increase over time, whereas P10 samples did not coagulate after 48 h (Table 4). In addition, the firmness (a_{30}) of the coagulum formed when testing P1 samples increased over time, whereas the firmness of the coagulum of P4 samples significantly decreased over time ($P = 0.01$). The increase in RCT is potentially due to the enzymatic degradation of caseins. The α_S-CN and β-CN constitute the basic microstructure of the curd, and reduced levels of those caseins results in slow curd formation and a soft coagulum as observed. Protease activity can affect the caseins to such an extent that coagulation of milk does not occur (St-Gelais and Hache, 2005; Amenu and Deeth, 2007), as observed in P10 samples. The proteolysis of caseins could result in curd shattering, high losses of fat in the whey, and reduced cheese yields (Fox et al., 2017). In relation to P1 samples, the rate of hydrolysis of α_S-CN and β-CN was lower and therefore the structure of the casein micelles was less affected (Lu et al., 2017). Consequently, the coagulum produced was firmer compared with other inoculated samples. The firmness measured in those samples was also higher than the firmness of control samples, possibly due to the higher rate at which κ-CN was hydrolyzed into para-κ-CN.

Finally, the inoculated samples had a similar TBC after 48 and 72 h of storage and still had different results in relation to proteolysis levels. Therefore, those results were mainly due to the protease activity added and not the bacterial count.

CONCLUSIONS

This study demonstrated that the protease produced by the psychrotrophic bacteria tested was thermoresistant and can affect the cheesemaking properties of milk. The rennet coagulation time and curd firmness were affected over storage time, as the protease was mainly active on essential caseins for the formation and structure of the coagulum (κ-CN and β-CN, respectively). The results indicated that certain levels of the protease tested can accelerate the coagulation of milk and produce a firmer coagulum, but increases in the level of protease-derived activity can result in a softer coagulum or a case where the coagulation of milk is not possible, due to extensive hydrolysis of α_S-CN and major whey proteins. Both situations may be undesirable in an industrial cheesemaking setting, as consistency

is required during production to obtain products with the same quality parameters. Therefore, this study highlighted the importance of controlling initial levels of psychrotrophs in milk, as the activity of their protease could affect the processability of milk over storage time. Further studies are necessary to determine the effect of those proteases on the manufacture and quality of cheese in an industrial scale. Additionally, as milk has a complex microbiota, it would be relevant to characterize other psychrotrophic bacterial proteases to determine their resistance to heat treatment and activity in milk.

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