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Effect of *Pseudomonas fluorescens* proteases on the quality of Cheddar cheese

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ABSTRACT

The objective of this study was to investigate the effect of adding different levels of a thermoresistant protease produced by a *Pseudomonas fluorescens* strain to milk on the manufacture and quality of Cheddar cheese. Fresh raw milk was collected, standardized, and pasteurized at 72°C for 15 s, and the enzyme was added to give a protease activity of 0.15 or 0.60 U/L (treatments P1 and P4, respectively), while one sample had no enzyme added (control). Milk was stored at 4°C for 48 h and Cheddar cheese was manufactured after 0 and 48 h of storage. Results indicated that the protease was active in milk during 48 h of storage; however, its effect on milk composition was minimal. The protein that was preferentially hydrolyzed by the protease over storage was β-casein, followed by κ-casein. The mean cheese yield and recovery of fat and protein obtained for all cheeses were not affected by protease activity. The protease showed low activity during cheese manufacture, possibly because of unfavorable conditions, including low pH. One of the factors that might have influenced protease activity was the pH of the curd (approximately 6.55 after acidification and 5.35 at milling), which was lower than that at which the enzyme would have optimum activity (pH 7 to 9). Consequently, the composition, pH, patterns of proteolysis, and hardness of all cheeses produced were similar and in accordance with values expected for that type of cheese, independently of the protease activity level. However, slight increases in proteolysis were observed in P4 cheeses and produced using milk stored for 48 h. Both the P1 and P4 cheeses had higher concentrations of free amino acids (FAA) compared with the control, whereas urea-PAGE electrophoretograms indicated a greater breakdown of caseins in the P4 cheese samples, which may be related to possible increases in numbers of proteolytic bacteria in milk during storage. Therefore, the thermoresistant psychrotrophic bacterial protease(s) tested in this study may affect the manufacture or quality of Cheddar cheese during ripening to a relatively limited extent. However, controlling initial levels of proteolytic bacteria in raw milk remains essential, because proteolysis affects the development of flavor and texture in cheese.
Key words: *Pseudomonas fluorescens*, psychrotrophic bacteria, thermoresistant protease, Cheddar cheese

INTRODUCTION

Worldwide production of cheese is approximately 19 $\times$ 10^6 tonnes per year, and production has increased at an average annual rate of approximately 4% over the past 30 yr (Fox et al., 2017). The increase in cheese consumption could be due to the positive dietary image of the product, convenience, and flexibility of use, as well as the great diversity of flavors and textures. Cheddar cheese is one of the most important cheese varieties worldwide and is one of the main dairy products exported by Ireland and the United Kingdom (Fox et al., 2017).

The quality of milk supplied for cheese manufacture is one of the main factors affecting the quality of the final product. The microbiota of raw milk during cold storage is mainly composed of gram-negative and gram-positive psychrotrophic bacteria. The most common psychrotrophs identified in raw milk belong to the genus *Pseudomonas* (Ercolini et al., 2009; Ribeiro Júnior et al., 2018). Most lipases and proteases produced by this bacterial species are thermoresistant and can withstand heating to 100°C for 30 min. Those enzymes are produced when psychrotrophic bacterial counts (**PBC**) are >6.0 or 7.0 $\log_{10}$ cfu/mL and are not eliminated after heat treatment, affecting the quality of cheese products (Fox, 1989; Fox et al., 2017). According to Sørhaug and Stepaniak (1997), PBC between 6.5 and 7.5 $\log_{10}$ cfu/mL can cause rancidity in

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hard cheeses, whereas counts between 7.5 and 8.3 $\log_{10}$ cfu/mL can cause off-flavors (i.e., rancidity and soapy taste) and reduced cheese yield. Most psychrotrophic proteases are metalloproteases, which preferentially hydrolyze κ-CN, then β-CN, and finally α_{S1}-CN, potentially causing reductions in cheese yield (Decimo et al., 2014). Boulares et al. (2011) and Mankai et al. (2012) observed decreases in cheese yield that were associated with the loss of protein into whey due to the activity of proteases produced by psychrotrophic bacteria. Even though certain products of microbial proteolysis can be undesirable in raw milk during storage and cheese manufacture, specific starter cultures are added into milk to promote proteolysis during ripening for the development of texture and flavor in different cheese varieties.

Several studies have investigated the effects of thermoresistant psychrotrophic bacterial proteases on UHT milk, determined the genetic characteristics of protease produced by *Pseudomonas*, and assessed their proteolytic activity (Marchand et al., 2009; Matéos et al., 2015; Caldera et al., 2016). Tye et al. (1988) evaluated the effects of a thermoresistant protease produced by a *Pseudomonas fluorescens* strain on the composition and sensorial characteristics of Cheddar cheese. However, the dynamics and activity of those proteases during the manufacturing process of a range of other dairy products, their effect on raw milk, and consequent implications for the quality of the final product require further investigations. Cheeses can vary greatly in relation to their processing parameters, pH, and microbiota, which can determine the type and activity of proteases in those products. Therefore, the aim of this study was to investigate the effect of different activity levels of a thermoresistant protease, produced by a *P. fluorescens* strain, on the manufacture, proteolysis, and quality of Cheddar cheese.

MATERIALS AND METHODS

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 72 h at 26°C with stirring at 90 rpm. Then, 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 (10% wt/vol). The inoculated reconstituted skim milk bottles were incubated for 120 h at 10°C with stirring at 90 rpm. Then, the bottles were centrifuged at 20,000 × *g* at 25°C for 30 min, and the supernatant containing protease was collected. The same supernatant containing the protease was used in a previous study conducted by the authors, and it was determined that the protease was thermoresistant, because it remained active in the supernatant after being heated at 72°C for 15 s (temperature applied during pasteurization; Paludetti et al., 2020).

Milk Collection

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. The milking equipment used and processes for udder preparation and sanitation were as described by Paludetti et al. (2018a). This experiment was repeated in triplicate over a 3-wk period. During that period, fresh raw milk was collected once per week and directly transferred from the milking machine line to 3 sanitized containers. The containers were transported to the Biofunctional Food Engineering facility at the Teagasc Food Research Centre (Moorepark, Cork, Ireland), where milk was standardized, pasteurized, and used for the production of Cheddar cheese.

Milk Standardization, Pasteurization, and Inoculation

Raw milk was skimmed using a tabletop cream separator (Milky FJ 130 ERR, Janschitz GmbH, Althofen, Austria), and the fat and protein contents in the separated cream and skim milk were measured by infrared absorption spectroscopy (MilkoScan FT6000, Foss Ireland Ltd., Dublin, Ireland). Pearson's square calculations were used to determine the amounts of skim milk and cream that should be mixed to achieve a protein-to-fat ratio of 0.95:1.00. The standardized milk was pasteurized at 72°C at a flow rate of 2 L/min with a holding time of 15 s using a Microthermics Lab heat exchanger (MicroThermics, Raleigh, NC).

The pasteurized milk was divided into 3 sanitized containers (40 L/container). In a previous study, the activity of the protease in the supernatant was determined by an azocasein test (0.030 U/mL·min, Paludetti et al., 2020) and, based on that value, the milk containers had different amounts of supernatant added to obtain different levels of protease activity in the milk samples. One unit of protease activity (U) was defined as the amount of enzyme required to hydrolyze azocasein resulting in an increase of 1 unit of absorbance per mL of sample (supernatant) per minute (Leighton et al., 1973). Supernatant was not added to one milk container used as a control (**C**), whereas 5

and 20 mL of supernatant per L of milk were added to the other 2 containers, to obtain activity levels of 0.15 and 0.60 U/L (4 × 0.15 U/L), respectively (treatments **P1** and **P4**, respectively). These concentrations were selected based on a previous study (Paludetti et al., 2020), in which milk samples containing those levels of protease activity coagulated after rennet addition, and therefore, Cheddar cheese manufacture would be possible. All milk containers were stored at 4°C for 48 h, and Cheddar cheese was produced after 0 and 48 h of inoculation. The experimental design of this study is shown in Figure 1.

Milk Composition

After 0 and 48 h of storage, the composition of each milk was assessed. The nitrogen (%N), noncasein nitrogen (**%NCN**) and NPN (%NPN) contents of the milk samples were determined using the Kjeldahl method, as described in the methods IDF 20-3 (IDF, 2004a), 29-1 (IDF, 2004b), and 20-4 (IDF, 2001), respectively, using a Tecator Digestor Auto and Kjeltec 8400 distiller (Foss Electric, Hillerød, Denmark). Noncasein protein (**%NCP**) was calculated by multiplying %NCN by 6.38. The pH of milk was also measured after 0 and 48 h of storage, before cheese manufacture.

The casein and whey protein fractions (κ-CN, α_{S2}-CN, α_{S1}-CN, β-CN, α-LA, β-LG A, β-LG B, and total casein) were identified and quantified (in triplicate) in the milk samples by HPLC as described by Paludetti et al. (2018b). Gradient elution and peak detection were performed according to the method of Mounsey and O'Kennedy (2009). The concentrations of each protein were determined by measurement of the peak areas with reference to standard of known concentrations.

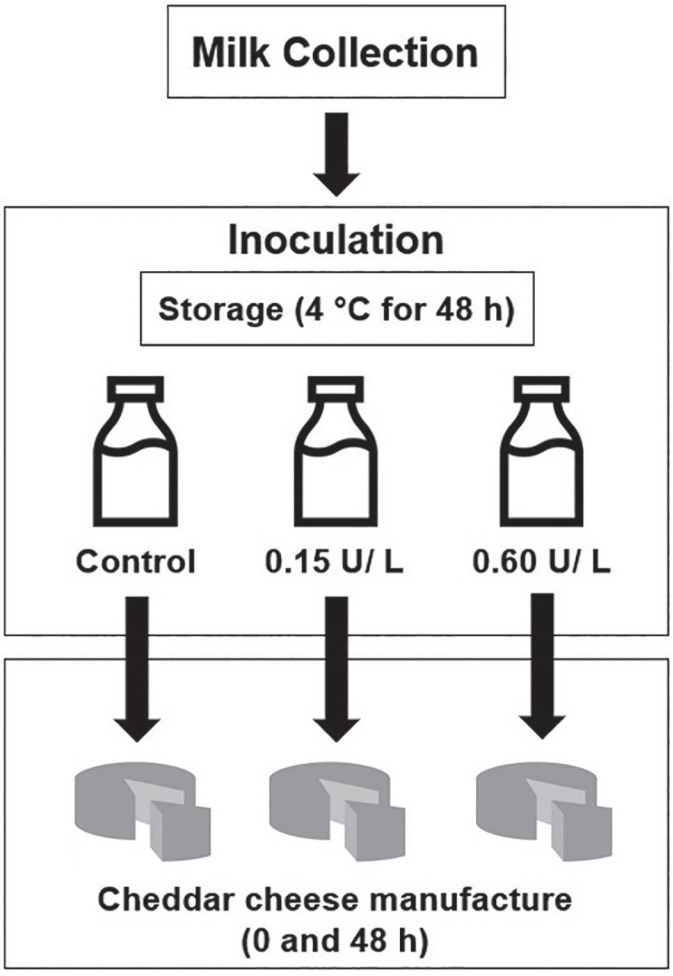

Figure 1. Experimental design showing process for production of cheeses made from milk inoculated with 0.15 (P1) and 0.60 (P4) U/L protease or not inoculated (control) and stored for 0 and 48 h before cheesemaking.

Microbiological Count in Milk Samples

The total bacterial count (**TBC**) and PBC were assessed in all milk samples at 0 and 48 h, after pasteurization and inoculation. Both bacterial counts were estimated using Petrifilm aerobic count plates (3M, Technopath, Tipperary, Ireland). Samples were incubated at 32°C for 48 h to determine TBC, and samples were incubated at 7°C for 10 d to determine PBC (Laird et al., 2004). Those tests were performed according to the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 2004).

Cheddar Cheese Production

After 0 and 48 h of inoculation and storage, milks with different protease activity levels were used to manufacture Cheddar cheese. During a 3-wk period, 2 cheesemaking trials were undertaken in each week. During each 3-wk period, 10 L of each milk was transferred into jacketed, stainless steel cheese vats, which contained automated variable speed cutting and stirring equipment. The milks from different treatments were inoculated at 32°C with strains of lactic acid bacteria (*Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *lactis*; R-604, Chr. Hansen Ltd., Cork, Ireland). After 60 min, rennet (Chy-Max Plus, Chr. Hansen Ltd.), diluted in Milli-Q water (approximately 0.1%, vol/vol), was added to each vat at a level of 2 mL/L.

Small-amplitude oscillatory rheometry (AR 2000ex, TA Instruments, New Castle, DE) was used to determine curd firmness (storage or elastic modulus, G′), by using a concentric-cylinder measuring geometry (cylindrical bob and cup). The dynamic changes in rheology during the coagulation process were monitored using a dynamic time sweep analysis, as described by Mateo et

al. (2010) and Lamichhane et al. (2018), but at 32°C. The gels were cut at a curd firmness of 35 Pa.

The cut program of 3 min consisted of alternating between cutting (40 s) and healing (20 s). The curd/whey mixture was stirred continuously for 15 min and then cooked; the temperature was increased at approximately 0.2°C/min from 32 to 38°C. At pH 6.15, the whey was drained and the curds were retained in the vat to promote further syneresis. The curds were inverted every 15 min and pH was monitored over that period (cheddaring). At pH 5.30, the curds were milled and salted (2.7% salt, wt/wt of cheese). The curds from each vat were weighed and molded in 2 × 500-g molds, pressed at 150 kPa overnight, weighed again, and vacuum-packed. The cheeses were stored at 4°C for 14 d and ripened at 8°C for 180 d.

Cheese yield (**Y%**) was calculated as the ratio between the weight of cheese produced (g) to the weight of milk used (g) multiplied by 100. Moisture-adjusted yield was not calculated because the moisture contents of the cheeses was not significantly different. Fat and protein recoveries (**%REC$_{FAT}$** and **%REC$_{PROT}$**, respectively) were calculated as the ratio of the weight (g) of the curd component (fat or protein) to the same component of milk (g) multiplied by 100.

Cheese and Whey Composition

At 7 d after manufacture, the moisture (IDF, 1982), protein (IDF, 1993), salt (IDF, 1988), and fat contents of all cheeses were determined. The fat content was measured by nuclear magnetic resonance, using a CEM Smart Trac System method (Cartwright et al., 2005). Based on the content of those parameters, the contents of TS, salt-in-moisture (**S/M**), moisture in the nonfat substance (**MNFS**), and fat in dry matter (**FDM**) were calculated. The pH of the cheeses was monitored after 7, 14, 90, and 180 d of ripening, by mixing 20 g of cheese with 12 g of deionized water (British Standards Institution, 1976). The fat and protein contents in whey were determined according to IDF standard methods 22 (IDF, 2008) and 20-3 (IDF, 2004a), respectively.

Assessment of Proteolysis

pH 4.6-Soluble N and Free Amino Acids. The pH 4.6-soluble N (**SN**) fractions of the cheeses ripened for 14, 90 and 180 d (expressed as % of total nitrogen, **TN**) were obtained as described by Fenelon and Guinee (2000). The nitrogen content of those fractions (**pH 4.6-SN/TN**) were determined in duplicate by the macro-Kjeldahl method (IDF, 1986). The levels of free amino acids (**FAA**) were measured as described by Sheehan et al. (2007). Equal volumes of pH 4.6-SN and trichloroacetic acid (240 g/L) were mixed to deproteinize the samples, and ion-exchange chromatography with post-column ninhydrin derivatization and visible colorimetric detection was used to separate the FAA.

Reversed-Phase HPLC. The peptide profiles of pH 4.6-soluble fractions were obtained by reversed-phase HPLC using an ultra-performance liquid chromatography system (**UPLC**) as described by McAuliffe (2017).

Urea-PAGE. Urea-PAGE was carried out on all cheeses after 14, 90, and 180 d of ripening. The analysis was performed using a Protean II xi cell vertical slab gel unit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), using a separating and stacking gel system, as described by Sheehan and Guinee (2004). Sodium caseinate powder (Kerry Ingredients, Listowel, Ireland), which contained α_{S1}-CN and β-CN, was used as a casein control, and was dissolved to give an equivalent concentration of protein. Gels were stained for 24 h with Instant Blue Coomassie (Expedeon/Abcam, Cambridge, UK) and scanned using a dual-lens Epson Perfection V700 Photo model J221A with Epson Scan software (Epson Deutschland GmbH, Meerbusch, Germany). The bands were identified as described by McSweeney et al. (1994) and Mooney et al. (1998).

Texture Analysis

The hardness of each cheese sample was measured after 14, 90, and 180 d of ripening. Cheese samples were prepared as described by Lamichhane et al. (2018). The texture profile was assessed using a TAHDi analyzer (Stable Micro Systems, Godalming, UK), equipped with a 75-mm (diameter) compression plate and a 50-kg load cell. Each cube was taken from the refrigerator and immediately compressed, in 2 successive bites, to approximately 40% of its original height at a rate of 1.00 mm/s (Henneberry et al., 2015). Hardness was calculated as described by Chevanan et al. (2006).

Statistical Analysis

The main effects of storage time and protease activity, as well as the interaction between storage time and protease activity, on the parameters measured in the inoculated milk were investigated. The least squares means of those effects were calculated using the GAUSSIAN procedure in SAS 9.3 (SAS Institute Inc., Cary, NC). The fixed effects included in each model were storage time (0 and 48 h) and protease activity (C, P1, and P4). The containers in which inoculated milk was stored within week were considered the experimental unit. The response variables were N%, NCN%, NPN%,

and NCP%; κ-CN, α_{S1}-CN, α_{S2}-CN, β-CN, α-LA, β-LG A and B, total casein contents; pH; TBC and PBC.

The influence of those same effects was also investigated on variables measured in the cheeses and whey produced. When the cheeses were considered the experimental unit, the response variables were Y%, $\%REC_{FAT}$, $\%REC_{PROT}$, and composition after 7 d (fat, protein, moisture, salt, TS, S/M, MNFS, FDM, and pH). When the whey produced was considered the experimental unit, the response variables were fat and protein contents.

Finally, we determined the influence of those same effects and ripening time (14, 90, and 180 d) on variables measured during the ripening of the cheeses produced (experimental unit). The least squares means of those effects were also calculated using the GAUSSIAN procedure in SAS 9.3 (SAS Institute Inc., Cary, NC). The response variables were pH, pH 4.6-SN/TN, concentrations of FAA, and hardness. For pH, the analysis also included the data measured at 7 d of ripening. Residual checks were made to ensure that the assumptions of the analyses were met. The Tukey test (at 5% error probability) was used to compare the means for all variables.

RESULTS

Milk Composition

The mean composition parameters and pH of milk from the different treatments are shown in Table 1, as well as the effects of storage time and protease activity and their interaction.

The mean (±SE) %N decreased significantly with increasing level of protease activity (C: 4.10, P1: 3.94 and P4: 3.91 ± 0.05%; Table 1). Protease activity also affected the %NCN and %NCP of milk samples. The variations in those fractions were considered minimal (Table 1).

In relation to casein fractions, results from the statistical analysis indicated that none of the factors significantly affected the level of κ-CN; however, numerical decreases in κ-CN levels with increasing level of protease added were observed (Table 1). The mean contents of β-CN, α-LA, β-LG A and B decreased slightly but significantly over storage time (Table 1).

The casein chromatograms of all milk samples, stored for up to 48 h, are shown in Figure 2. The β-CN content decreased with increasing level of protease added. Storage time affected the pH of the milk samples; however, the decreases observed over time were minimal.

Microbiological Counts in Milk

The mean TBC and PBC measured at 0 and 48 h in all milk samples, as well as the effects of storage time, protease activity, and their interaction, are shown in Table 2. The mean TBC increased significantly with protease activity and storage time, whereas PBC increased significantly with storage time; it is noted that the enzyme preparation used was not pasteurized and so may have contained live cells.

Table 1. Mean (±SE) contents of nitrogen fractions, caseins and major whey proteins, and pH, in control (C) milk samples and milk samples with protease activity levels of 0.15 (P1) and 0.60 (P4) U/L stored for up to 48 h, and significance of the main effects of storage time (S), protease activity (P), and their interaction

	Protease activity							Significance		
	C		P1		P4					
Component	0 h	48 h	0 h	48 h	0 h	48 h	SE	S	P	S × P
Nitrogen fractions[1]										
%N	4.17	4.20	4.01	3.87	3.90	3.93	0.09	0.80	0.04	0.68
%NCN	0.14	0.14	0.14	0.14	0.15	0.15	0.004	0.39	0.04	0.89
%NPN	0.04	0.03	0.03	0.04	0.04	0.04	0.001	1.00	0.07	0.30
%NCP	0.87	0.88	0.89	0.92	0.97	1.00	0.03	0.45	0.01	0.95
Caseins and whey proteins (μg/μL)										
κ-CN	7.68	6.97	6.75	6.35	5.97	5.82	0.34	0.09	0.06	0.56
α_{S2}-CN	5.15	4.72	4.61	4.50	4.82	4.63	0.20	0.30	0.42	0.12
α_{S1}-CN	15.03	14.10	14.02	13.92	15.22	14.33	0.38	0.83	0.32	0.05
β-CN	15.12	14.15	14.05	12.74	14.10	11.63	0.38	0.0002	0.05	0.05
α-LA	1.24	1.06	1.08	1.04	1.11	1.07	0.06	0.03	0.52	0.20
β-LG A	2.76	2.37	2.37	2.36	2.47	2.37	0.11	0.03	0.42	0.08
β-LG B	3.12	2.67	2.75	2.63	2.90	2.74	0.11	0.01	0.41	0.12
Total casein	50.10	46.05	45.64	43.56	45.52	43.67	1.50	0.01	0.22	0.49
pH	6.60	6.58	6.59	6.58	6.61	6.59	0.01	0.005	0.24	0.61

[1]%NCN = noncasein nitrogen; %NCP = noncasein protein.

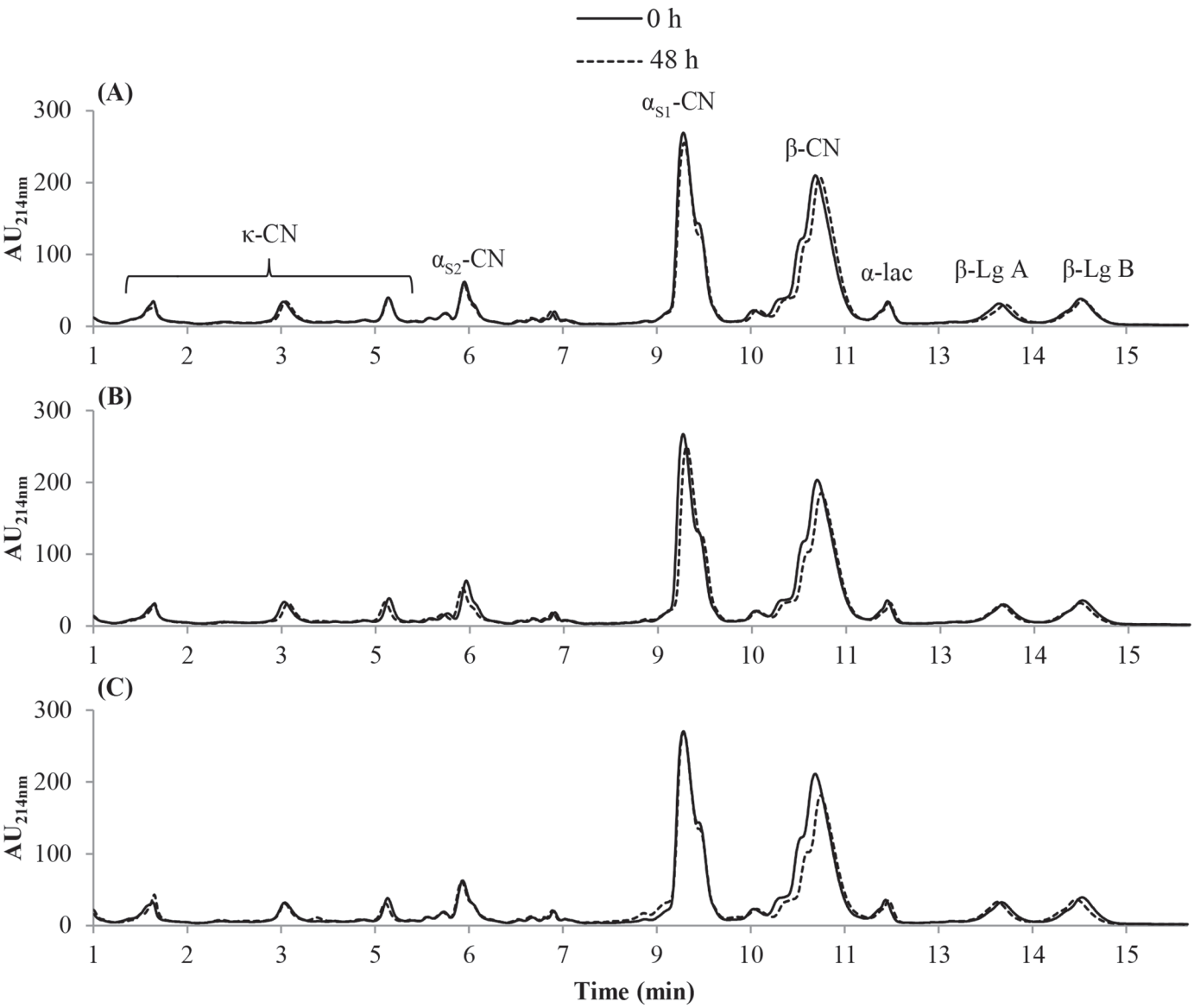

Figure 2. Separation of bovine milk caseins, β-LG, and α-LA by reversed-phase HPLC (measured in absorbance units, AU). Representative chromatograms of (A) control milk samples and samples with protease activity levels of (B) 0.15 and (C) 0.60 U/L stored for up to 48 h are shown.

Table 2. Mean (±SE) total (TBC) and psychrotrophic (PBC) bacterial counts measured in control (C) milk samples and samples with protease activity levels of 0.15 (P1) and 0.60 (P4) U/L stored for up to 48 h, and significance of the main effects of storage time (S), protease activity (P), and the interaction between them

	Protease activity							Significance		
	C		P1		P4					
Bacterial count (log_{10} cfu/mL)	0 h	48 h	0 h	48 h	0 h	48 h	SE	S	P	S × P
TBC	2.21	3.27	2.41	4.32	3.41	5.00	0.34	<0.0001	0.04	0.13
PBC	2.13	3.32	3.25	4.51	4.02	4.78	0.54	0.007	0.17	0.73

Table 3. Mean (±SE) cheese yield (Y%), fat recovery (%REC_{FAT}), and protein recovery (%REC_{PROT}) measured in control (C) milk samples and samples with protease activity levels of 0.15 (P1) and 0.60 (P4) U/L stored for up to 48 h, and significance of the main effects of storage time (S), protease activity (P), and the interaction between them

	Protease activity							Significance		
	C		P1		P4					
Variable	0 h	48 h	0 h	48 h	0 h	48 h	SE	S	P	S × P
Y%	11.40	11.94	11.56	11.48	11.38	11.17	0.1	0.66	0.13	0.27
%REC_{FAT}	86.73	85.91	86.93	85.24	86.85	85.15	0.5	0.03	0.87	0.72
%REC_{PROT}	72.23	70.16	76.54	75.76	70.84	70.76	1.1	0.54	0.68	0.37

Cheese Yield and Cheese and Whey Composition

The mean Y%, %REC_{FAT}, and %REC_{PROT} obtained for all cheeses manufactured after 0 and 48 h of milk storage are shown in Table 3. Storage time affected the mean %REC_{FAT} ($P = 0.03$), whereas none of the factors affected the mean Y% or %REC_{PROT}.

The effects of storage time, protease activity, and interaction between those factors on cheese and whey composition are shown in Table 4. Only FDM content was affected by storage time. None of the factors influenced the fat and protein contents in whey.

The mean (±SE) pH measured in the cheese samples from the different treatments and stored for 7, 14, 90, and 180 d are shown in Table 5. Ripening time ($P = 0.01$) and treatment ($P < 0.001$) affected the pH of the cheeses. Storage time ($P = 0.05$) and the interaction between ripening time and storage time ($P = 0.96$), ripening time and treatment ($P = 0.99$), storage time and treatment ($P = 0.10$), and ripening time, storage time, and treatment ($P = 0.99$) did not affect the mean pH of the samples.

Proteolysis During Cheese Ripening

The mean %pH 4.6-SN/TN obtained for cheeses C, P1, and P4 throughout ripening time are shown in Table 6. Ripening time affected the mean N content quantified in those fractions ($P < 0.0001$); however, the level of added protease ($P = 0.17$), storage time ($P = 0.71$), or their interactions did not affect mean N content.

The concentrations of all FAA increased significantly over ripening time, with the exception of Lys (Table 7). The mean concentrations of the FAA measured in all cheeses after 180 d of ripening are shown in Figure 3. Leucine and Glu were the most abundant FAA quantified in all cheeses produced using milk stored for 0 and 48 h, with approximately 250 and 235 mg/kg at 180 d, respectively, followed by Phe, Val, Lys, His, and Pro (approximately 190, 110, 93, 90, and 74 mg/kg of cheese, respectively).

The peptide peaks obtained for the pH 4.6-soluble fractions of the cheeses at 180 d of ripening were broadly similar (Figure 4). Differences were observed

Table 4. Mean (±SE) composition of cheeses produced using control (C) milk and milk with activity levels of 0.15 (P1) and 0.60 (P4) U/L, and fat and protein contents in whey obtained during manufacture of Cheddar cheese; and significance of the main effects of storage time (S), protease activity (P) and the interaction between them

	Protease activity							Significance		
	C		P1		P4					
Item[1]	0 h	48 h	0 h	48 h	0 h	48 h	SE	S	P	S × P
Cheese										
Fat (%)	32.08	31.58	31.29	31.46	31.49	31.85	0.30	0.97	0.34	0.50
Protein (%)	26.38	25.66	23.81	25.57	24.37	24.99	0.71	0.47	0.16	0.43
Moisture (%)	34.76	35.82	36.00	36.21	35.74	35.69	0.51	0.39	0.41	0.62
Salt (%)	1.39	1.45	1.28	1.29	1.42	1.31	0.06	0.83	0.21	0.50
TS (%)	65.24	64.18	64.00	63.79	64.26	64.31	0.51	0.39	0.41	0.62
SM (%)	4.03	4.06	3.55	3.57	3.99	3.69	0.23	0.69	0.22	0.75
MNFS (%)	51.16	52.33	52.39	52.83	52.16	52.36	0.53	0.22	0.43	0.68
FDM (%)	49.18	49.30	48.89	49.32	49.01	49.54	0.12	0.04	0.10	0.47
Whey										
Fat (%)	0.32	0.35	0.32	0.32	0.31	0.35	0.02	0.31	0.82	0.67
N (%)	0.92	1.06	0.96	1.18	0.92	1.22	0.09	0.09	0.47	0.84

[1]SM = salt-in-moisture; MNFS = moisture in the nonfat substance; FDM = fat in dry matter.

Table 5. Mean (±SE) pH measured throughout ripening of Cheddar cheeses produced using control (C) milk and milk with protease activity levels of 0.15 (P1) and 0.60 (P4) U/L and stored for up to 48 h

Protease activity	Time (h)	Ripening time (d)			
		7	14	90	180
C	0	5.19 ± 0.30	5.20 ± 0.29	5.25 ± 0.26	5.29 ± 0.30
	48	5.05 ± 0.01	5.08 ± 0.00	5.11 ± 0.00	5.15 ± 0.00
P1	0	4.95 ± 0.01	5.00 ± 0.05	5.06 ± 0.05	5.11 ± 0.05
	48	5.10 ± 0.27	5.13 ± 0.27	5.18 ± 0.28	5.23 ± 0.28
P4	0	5.17 ± 0.19	5.18 ± 0.20	5.20 ± 0.22	5.25 ± 0.21
	48	5.17 ± 0.26	5.19 ± 0.30	5.20 ± 0.28	5.26 ± 0.26

in relation to the size of the peptide peaks between samples, indicating possible quantitative, but not qualitative, differences in the levels of proteolysis in terms of relative concentrations of those peptides.

Electrophoresis indicated a progressive increase in the breakdown of α_{S1}-CN and β-CN throughout ripening (Figure 5). The pattern of proteolysis over ripening was similar between cheeses manufactured using uninoculated and inoculated milk after 0 h of storage. In contrast, we detected an increase in the breakdown of β-CN with increasing levels of protease activity in cheeses manufactured using milk stored with enzyme added for 48 h. In addition, the electrophoretogram for sample P4 manufactured after 48 h of milk storage showed an increase in the intensity of the band denoted by X. This indicates an increase in proteolytic activity in that samples compared with C and P1.

Ripening time affected the mean hardness of the cheeses (14 d: 265.9, 90 d: 225.6, 180 d: 127.7 ± 10.3 N; $P < 0.0001$; Table 8). Storage time ($P = 0.54$), protease activity ($P = 0.13$), ripening time × treatment ($P = 0.73$), storage time × treatment ($P = 0.61$), and ripening time × storage time × treatment ($P = 0.98$) did not affect mean cheese hardness.

DISCUSSION

Overall, the addition of the *Pseudomonas* protease after milk pasteurization had an effect both in milk during storage and, to a lesser extent, in cheese during ripening. The concentrations of some caseins decreased in the milk during cold storage with increasing levels of protease activity, indicating that the protease was active during this phase. The addition of protease seemed to have mainly affected the mean β-CN and κ-CN contents in milk over storage time (Table 1 and Figure 2). Differences of approximately 1.5 and 2.0 μg/μL in mean κ-CN and β-CN concentrations, respectively, were observed between samples C and P4. Similarly, Baglinière et al. (2013) reported that a thermoresistant protease (AprX) produced by a *P. fluorescens* strain preferably hydrolyzed β-CN in milk, and Matéos et al. (2015) observed rapid hydrolysis of κ-CN and β-CN in milk in similar proportions by AprX. However, other studies (Zhang et al., 2015, 2018) reported that thermoresistant proteases produced by *Pseudomonas* strains preferably hydrolyze κ-CN followed by β-CN. Those differences in results could be due to the methodology used to investigate hydrolysis or differences in the specificity of the protease isolated. β-Casein, along with α_S-CN, constitutes the basic microstructure of cheese, and their reduced concentrations in milk could affect rennet clotting of milk and curd formation (St-Gelais and Hache, 2005).

In addition, the increase in breakdown of caseins and whey proteins in milk with increasing protease activity over storage time before cheesemaking could have resulted in loss of AA or peptides into whey, which could

Table 6. Mean (±SE) levels of pH 4.6-soluble N/total N (g/100 g) measured during ripening of cheeses produced with control (C) milk and milk with protease activity levels of 0.15 (P1) and 0.60 (P4) U/L and stored for up to 48 h

Protease activity	Time (h)	Ripening time (d)		
		14	90	180
C	0	4.68 ± 1.33	14.74 ± 1.74	20.55 ± 0.56
	48	6.31 ± 0.23	17.45 ± 0.32	22.53 ± 0.56
P1	0	5.35 ± 1.92	16.55 ± 3.68	22.58 ± 4.07
	48	5.58 ± 0.97	15.97 ± 1.04	21.35 ± 0.71
P4	0	5.60 ± 1.24	16.74 ± 1.70	22.71 ± 0.66
	48	5.92 ± 1.71	15.99 ± 2.10	22.05 ± 1.13

have resulted in the increase in %N in whey samples over time as shown in Table 4 (Emmons et al., 1990; Barbano et al., 1991). We also observed decreases in $\%REC_{PROT}$ with increasing amount of protease added (Table 3), which could be due to the breakdown of proteins in milk during storage.

The increases in TBC and PBC with protease activity and over storage could be due to the *P. fluorescens* cells in the supernatant (containing protease) used for inoculation; the initial TBC and PBC increased as the amount of supernatant added was increased to achieve the experimental levels of protease activity in the milk samples.

Although the level of protease activity and storage time had little effect on the composition of milk and cheese, the statistical differences observed in the mean Y%, $\%REC_{FAT}$, $\%REC_{PROT}$, and whey composition were also minimal (Tables 3 and 4). Those results could also indicate that the protease had a low activity when subjected to the processing conditions (e.g., variations in pH). In addition, the composition of the cheeses produced was similar to that obtained by Auldist et al. (2016) (moisture: 37.5%, fat: 31%, protein: 25.5%, salt: 1.70%, SM: 4.60%, FDM: 49.5%) and reported by Fox et al. (2017) (moisture: 37.2%, protein: 25.4%, fat: 33.1%, salt: 1.80%, SM: 4.8%).

The variations in mean pH between treatments were small. After 7 d of ripening, the mean pH of cheeses from each treatment varied from 4.95 to 5.15, which was within the ranges specified for premium quality Cheddar as defined by Fox (1975) and Pearce and Gilles (1979). The pH of the cheeses increased by approximately 0.1 unit after 180 d of ripening, similar to the increase reported by Fox et al. (2004). The increase in pH during ripening is due to formation of alkaline nitrogenous compounds and catabolism of lactic acid (Fox et al., 2004).

The pattern of proteolysis investigated in each experimental cheese (quantification of pH 4.6 SN/TN, concentrations of FAA, peptide profiles, urea-PAGE) indicated that the protease added had minimal effect on proteolysis during ripening.

The quantification of the pH 4.6 SN/TN is a measurement of proteolysis in cheeses, because this fraction contains the peptides resulting from casein breakdown (Hou et al., 2014). The magnitude of the increase in pH 4.6 SN/TN over ripening (from approximately 5.0% at 14 d to 22.0% at 180 d; Table 6) was similar between all cheeses and in agreement with previous reports for Cheddar cheese (Fenelon et al., 2000a; Wang et al., 2011; Hou et al., 2014). The progressive breakdown of the casein matrix during ripening also increases concentrations of FAA (Fox and McSweeney, 1996), as was observed in all samples of pH4.6-SN extracts of cheese in this study (Figure 3). Fenelon et al. (2000b) and McCarthy et al. (2017) also observed high levels of Glu, His, Leu, Lys, Phe, Pro, and Val in Cheddar cheeses.

Storage time did not affect the concentrations of each FAA in cheese; however, we observed some trends in relation to ripening and protease activity. Cheeses P1 and P4, which were produced using milk stored for 48 h, had higher concentrations of Ala, Glu, Gly, Ile, Leu, Lys, Met, Pro, and Val than the control cheeses. These increases in proteolysis in the P1 and P4 cheeses

Table 7. Significance of the main effects of storage time (S), protease activity (P), ripening time (R), and their interactions on the content of free AA contents (mg/kg of cheese) measured in the pH 4.6-soluble fractions of control cheese samples and cheeses manufactured with milk containing 0.15 or 0.60 U/L of protease activity

AA (mg/kg)	Significance					
	S	P	R	S × P	R × P	S × P × R
Asp	0.21	0.52	0.0007	0.26	0.60	0.27
Thr	0.60	0.49	0.002	0.37	0.24	0.13
Ser	0.54	0.67	0.01	0.39	0.67	0.59
Glu	0.85	0.84	0.03	0.28	0.92	0.34
Gly	0.91	0.83	0.02	0.37	0.59	0.21
Ala	0.77	0.81	0.006	0.36	0.33	0.11
Cys	0.11	0.18	<0.0001	0.95	0.74	0.89
Val	0.96	0.86	0.008	0.34	0.58	0.19
Met	0.98	0.82	0.0002	0.32	0.65	0.18
Ile	0.60	0.89	0.007	0.11	0.81	0.64
Leu	0.64	0.94	<0.0001	0.09	0.80	0.47
Tyr	0.20	0.20	<0.0001	0.50	0.45	0.85
Phe	0.60	0.47	<0.0001	0.55	0.40	0.77
His	0.41	0.54	<0.0001	0.25	0.59	0.70
Lys	0.80	0.95	0.07	0.45	0.67	0.18
Trp	0.30	0.73	<0.0001	0.19	0.71	0.69
Arg	0.31	0.32	<0.0001	0.51	0.50	0.83
Pro	0.21	0.52	0.0007	0.26	0.60	0.27

could be related to the increase in bacterial counts with protease activity that was observed in milk samples. Bacterial cells that remained in the milk after pasteurization or *Pseudomonas* cells that were present in the supernatant could have contributed to an increased concentration of enzymes in milk during storage, increasing the proteolytic activity in the cheese produced with that milk. Concentrations of FAA are associated with the development of flavor in cheese (Fox et al., 2017), and the prolonged storage of milk containing high levels of proteolytic bacteria could affect the sensory quality of the product.

Urea-PAGE electrophoretograms showed a slight increase in proteolysis in P4 cheese samples manufactured after 48 h of milk storage; the increase in intensity of band X, above β-CN, was greater in P4 cheeses compared with samples C and P1 (Figure 5). Furthermore, the proteolysis patterns of all samples of cheese pH4.6-SN extracts were similarly independent of the level of protease activity. During ripening, α_S-CN is degraded more extensively than β-CN into smaller fragments by proteolytic enzymes (St-Gelais and Hache, 2005), as we also observed in the electrophoretograms.

In a previous study, we observed that the protease (or proteases) tested potentially has a specificity similar to that of chymosin in relation to casein hydrolysis (Paludetti et al., 2020). This could explain the similarity between the peptide peaks obtained for control cheese

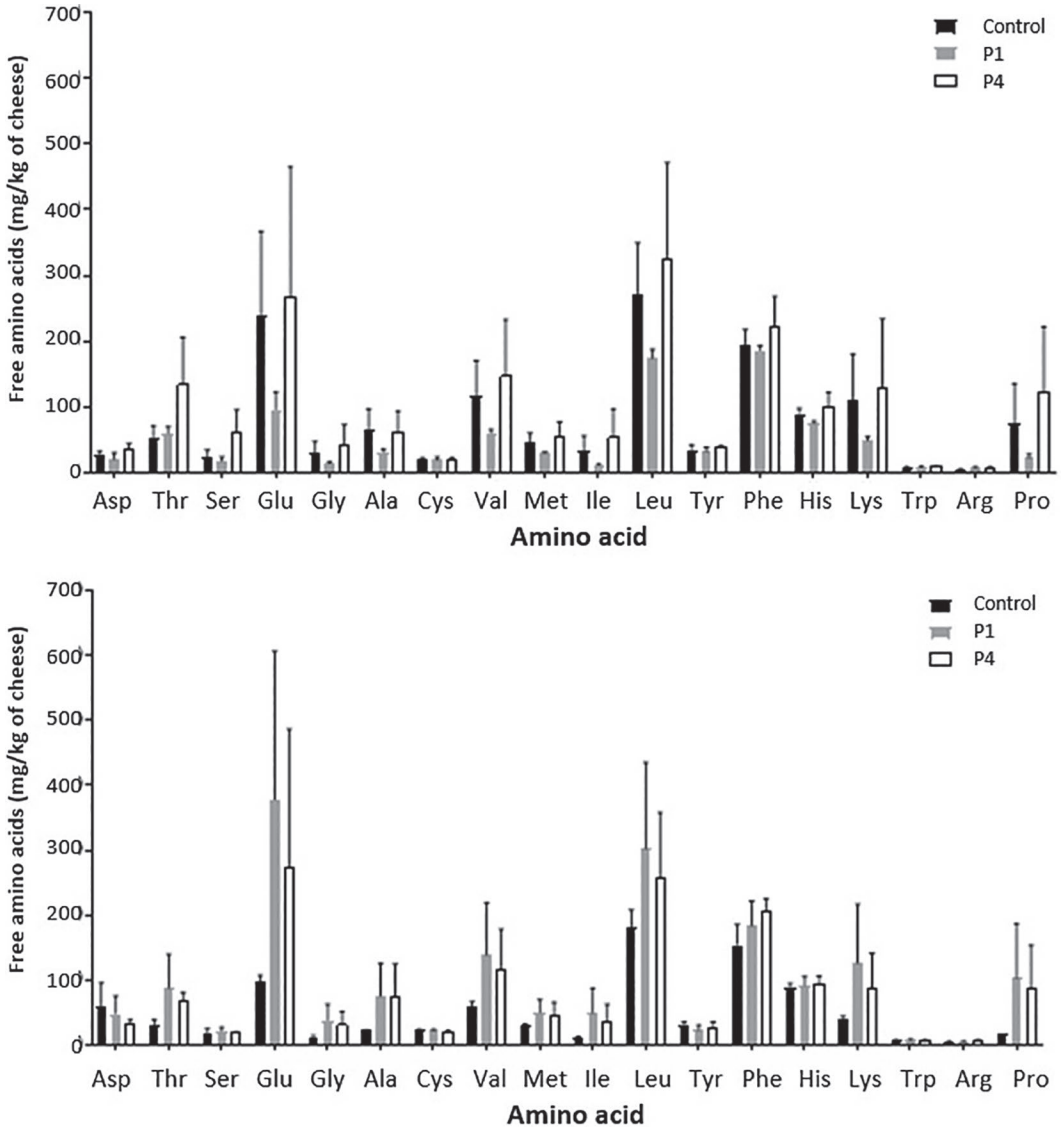

Figure 3. The effect of milk treatments on the mean levels of individual free AA in pH 4.6-soluble N extracts from Cheddar cheeses at 180 d of ripening. Cheeses were produced after (A) 0 h and (B) 48 h of storage using control milk or milk inoculated with 0.15 (P1) and 0.60 (P4) U/L protease. Data presented are means of data from 3 replicate trials. Error bars show the SEM from 3 replicate trials.

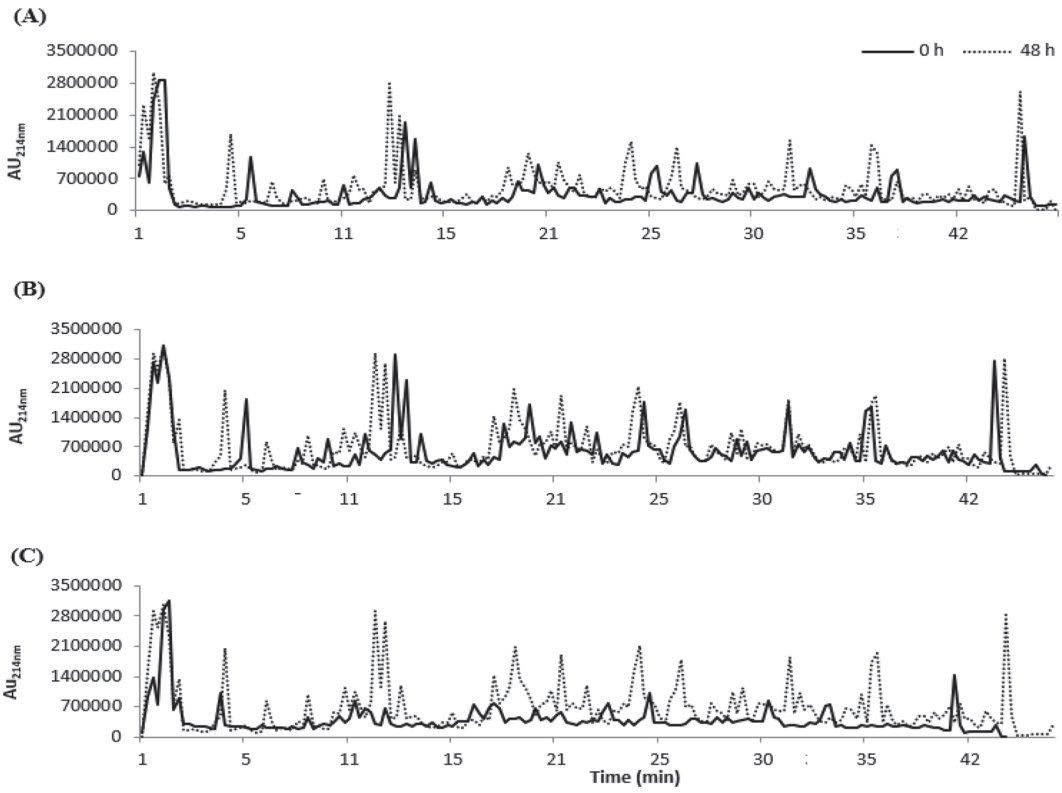

Figure 4. Representative reversed-phase ultra-performance liquid chromatography profiles of peptides in the pH 4.6-soluble fraction of Cheddar cheeses manufactured using milk with protease activity levels of (A) 0 (control), (B) 0.15, and (C) 0.60 U/L, after 0 and 48 h of storage, at 180 d of ripening.

samples and samples with added protease(s) at 180 d of ripening (Figure 4). In addition, the decrease in some of the peptide peaks could indicate that those could have been further hydrolyzed by the protease during ripening.

Proteolysis is a determining factor of the final texture and flavor in cheeses (Fox et al., 2017). Because levels of proteolysis were similar between samples, it is perhaps not surprising that the mean hardness values obtained for cheeses from each treatment were similar. The softening of cheese texture during ripening is a result of the hydrolysis of the casein matrix, solubilization of calcium in the early stages of ripening, and a decrease in the water activity of the curd (McSweeney, 2004; Fox et al., 2017). The latter occurs as a consequence of changes in water-binding by the carboxylic acid and amino groups formed on hydrolysis. Therefore, as expected, the hardness of the cheese samples decreased over ripening time. The chemical composition of cheese (e.g., fat and moisture content) can also affect the hardness of cheese during ripening. In addition, as reported by Lamichhane et al. (2019), hydrolysis of α_S- and β-CN influences the fracture stress and strain of cheeses, respectively. The decreases in hardness between 14 and 180 d of ripening (approximately 100 N) observed in this study were similar to those reported by McCarthy et al. (2016) and Hickey et al. (2018) for Cheddar-style cheeses.

Finally, the results suggested that the protease was not extensively active in the Cheddar cheese matrix. An azocasein test was carried out, as described by Paludetti et al. (2020), to investigate whether the protease was transferred into the whey during manufacture of each cheese. The results of the test were not significantly different (0 h; C: 0.065 ± 0.064 nm, P5: 0.075 ± 0.066 nm, P20: 0.092 ± 0.095 nm; 48 h, C: 0.095 ± 0.119 nm, P5: 0.174 ± 0.045 nm, P20: 0.147 ± 0.205 nm; $P > 0.05$), indicating that the protease remained in the cheese matrix. The activity of thermoresistant proteases produced by *Pseudomonas* bacteria is optimal at a pH between 7 and 9 and in a temperature range of 30 to 45°C (Martins et al., 2015). Therefore, the low pH and storage temperature of the cheeses could have affected protease activity. Other cheese types are characterized by higher ripening temperatures and pH, such as Swiss cheese (stored at approximately 22°C for 4 to 6 wk), Mozzarella cheese (heated to 57°C during stretching), and surface mold-ripened cheeses (e.g., Camembert of pH ~7.0; Fox et al., 2017), and may be

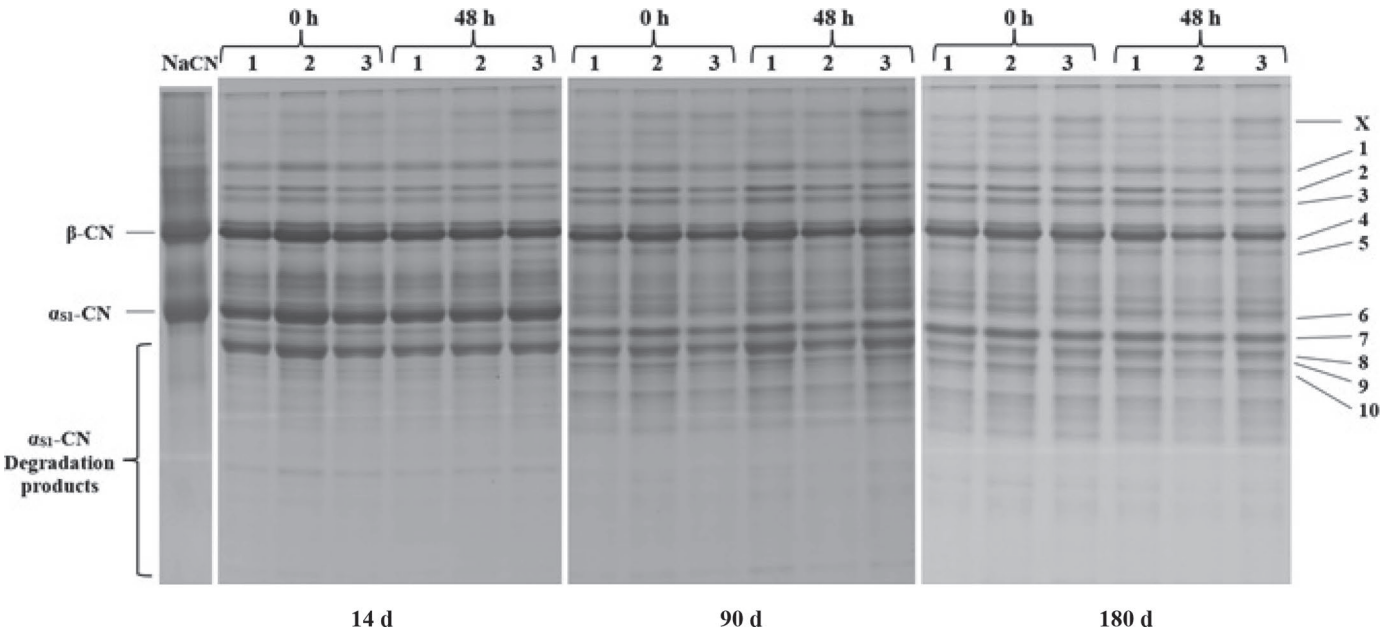

Figure 5. Urea-polyacrylamide gel electrophoretograms of Cheddar cheeses stored for 14, 90, and 180 d. Cheeses were produced using control (C) milk or milk inoculated with 0.15 (P1) and 0.60 (P4) U/L protease (lanes 1, 2, and 3, respectively) after 0 and 48 h of storage. Sodium caseinate (lane NaCN), loaded at an equivalent weight of protein (4.75 mg per lane) was included as an unhydrolyzed casein control. Protein bands were identified according to Mooney et al. (1998) and McSweeney et al. (1994): 1 = β-CN f(106–209) (γ2); 2 = β-CN f(29–209) (γ1); 3 = β-CN f(108–209) (γ3); 4 = β-CN; 5 = β-CN f(1–192); 6 = α_{S1}-CN; 7 = α_{S1}-CN f(102–199); 8 = α_{S1}-CN f(24–199); 9 = α_{S1}-CN f(121–199); 10 = α_{S1}-CN f(33-*). The band that increased in intensity with protease concentration is denoted by X.

more significantly affected if protease is active in the matrix.

CONCLUSIONS

The levels of activity of the *Pseudomonas* protease or proteases tested had a minimal effect on proteolysis levels in milk and Cheddar cheese produced. Processing and storage conditions of milk and cheese could have influenced the activity of the protease; consequently, the manufacture and quality of the Cheddar cheeses produced were not affected. Independent of the protease activity level, the composition and levels of proteolysis during ripening in all cheeses were similar and as expected. The levels of protease activity tested did not affect the quality of whey, a valuable by-product obtained in cheese manufacture. Therefore, the levels of psychrotrophic bacterial protease(s) tested in milk during storage did not contribute to increased proteolysis in Cheddar cheese. However, levels of proteolytic activity in milk that are higher than those tested in this study could affect proteolysis in cheese during ripening. Other cheese types are characterized by higher ripening temperatures and pH, and may be more significantly affected if the protease is active in the matrix. Further studies may be necessary to determine the effects of higher protease concentrations, as well as their effects on other cheese types.

Table 8. Mean (±SE) hardness (N) measured during ripening of Cheddar cheeses produced with control (C) milk and milk with protease activity levels of 0.15 (P1) and 0.60 (P4) U/L stored for up to 48 h

Protease activity	Time (h)	Ripening time (d)		
		14	90	180
C	0	258.7 ± 51.7	226.5 ± 88.6	121.2 ± 28.0
	48	225.1 ± 51.8	211.0 ± 16.0	108.1 ± 13.6
P1	0	248.5 ± 76.4	210.4 ± 48.7	122.6 ± 40.4
	48	242.0 ± 90.1	221.6 ± 33.0	119.1 ± 13.4
P4	0	281.9 ± 97.5	225.1 ± 43.4	127.6 ± 19.7
	48	314.8 ± 125.0	244.9 ± 43.1	134.7 ± 36.0

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