ABSTRACT

There is growing interest in process cheese in many developing countries due to its versatility and shelf stability. The main structural component (base) of most processed cheese formulations is young Cheddar cheese that has high levels of intact casein (CN). Exporting natural Cheddar cheese base from the US to distant overseas markets would require the aging process to be slowed or reduced. As Cheddar cheese ripens, the original structure is broken down by proteolysis and solubilization of insoluble calcium phosphate. We explored the impact of varying rennet levels (we also used a less proteolytic rennet) and application of high-pressure processing (HPP) to Cheddar cheese, as we hoped these treatments might limit proteolysis and concomitant loss of intact CN. To try to retain high levels of insoluble calcium, all experimental cheeses were made with a high draining pH and from concentrated milk. To compare our intact CN results with current practices, we manufactured a Cheddar cheese that was prepared according to typical industry methods, i.e., use of unconcentrated milk, calf chymosin (higher levels), and low draining pH value (~6.2). All experimental cheeses were made from ultrafiltered milk with protein and casein contents of ~5.15% and 4.30%, respectively. Three (low) rennet levels were used; control (38 IMCU/mL rennet/250 kg milk), 25% and 50% reduced from this level. All experimental cheeses had similar moisture contents (~37%) and total calcium levels. Four d after cheese was made, half of the experimental samples from each vat underwent HPP at 600 MPa for 3 min. Cheddar cheese functionality was monitored during aging for 240 d at 4°C. Cheddar cheese base was used to prepare process cheese after aging for 14, 60, 120, 180, and 240 d. Loss tangent (LT) values of cheese during heating were measured by small strain oscillatory rheology. Intact CN levels were measured using Kjeldahl method. Acid/base titrations were used to determine the buffering capacity and insoluble Ca levels as a percentage of total calcium. The LT_max values (an index of meltability) in process cheese increased with aging for all the cheese bases; HPP treatment significantly decreased LT_max values of both base (natural) and process cheeses. All experimental cheeses had much higher levels of intact CN compared with typical industry-make sample. Process cheese made from the experimental treatments had visually higher stretching properties than process cheese made from Cheddar with the typical industry make procedure. Residual rennet activity was not impacted by rennet level, but the rate of proteolysis was slightly slower with lower rennet levels. HPP treatment of Cheddar cheese reduced residual rennet activity and decreased the reduction of intact CN levels. HPP treatment of Cheddar cheese resulted in process cheeses that had slightly higher hardness values, lower LT_max values and retained higher storage modulus values at 70°C. We also observed that the other make procedures we used in all experimental treatments (i.e., use of a less proteolytic chymosin, concentrated cheese milk, and maintaining a high draining pH value) had a major impact on retaining high levels of intact CN.

INTRODUCTION

Block and sliced process cheese with good sliceability and elasticity require high levels of intact CN (e.g., 70–90%), which is usually found in young cheese (i.e., the base) aged less than 4 mo (Meyer, 1973; Guinee et al., 2004). Young cheese has a dense network of interconnected CN-CN interactions and high levels of insoluble (INSOL) calcium phosphate that help provide structure. However, as the natural cheese ages, this structure is broken down by a combination of proteolysis of the CNs and solubilization of INSOL calcium phosphate (Lucey et al., 2003). This structural loss in the cheese base impacts the texture and rheological properties of the process cheese, such as, decreasing the hardness and increasing the meltability (Kapoor and Metzger, 2008).
The US dairy industry is interested in increasing its cheese exports, which are currently about 6% of its total production (USDEC, 2021). This change could include more exports of natural cheese base to supply overseas process cheese manufacturers. Shipments of natural cheese base to these distant markets could take several months during which significant breakdown of the structure would occur. Since young cheese base is the major ingredient of process cheese, strategies are needed to reduce the rate of protein breakdown as well to retain high levels of INSOL calcium phosphate.

Ultrafiltered (UF) milk is widely used in cheesemaking to increase cheesemaking yield (Green et al., 1981). The UF process concentrates the fat and proteins in milk while allowing the lactose and soluble components to permeate. Increasing the protein and fat contents of cheesemilk by UF increases the amount of cheese produced (yield) but there have been some concerns that the resultant cheese might be firmer and slower to ripen than cheese made from unconcentrated milk (Green et al., 1981; Creamer et al., 1987). However, adjustments can be made to the cheesemaking process so that cheeses made from UF concentrated milk (<5% protein) are similar in quality to control cheeses (Govindasamy et al., 2004, 2005). In this study, we used a UF concentrated cheese milk to help produce a firmer body and possibly slow down proteolysis.

During cheesemaking, much of the soluble Ca is lost in the whey at drainage, while the INSOL calcium associated with the CNs is mostly retained in the protein matrix of the cheese (Johnson and Lucey, 2006). The amount and extent of Ca loses during cheesemaking is influenced by the acidity of milk at rennet coagulation, with lower pH values increasing its solubilization (Lucey and Fox, 1993). Ongoing solubilization of INSOL Ca occurs due to increased acidity during the cheesemaking process as well as further solubilization during storage (Hassan et al., 2004). In this study, we used a high drain pH to help retain more INSOL Ca crosslinking in our Cheddar cheeses.

Residual rennet retained in the cheese after whey drainage is responsible for much of the primary proteolysis during the Cheddar aging process (McSweeney, 2004). Traditionally, calf chymosin has been used in Cheddar cheesemaking. Camel chymosin has also been found to be a suitable substitute for calf chymosin (Bansal et al., 2009; Govindasamy et al., 2010). Camel chymosin has a narrow specificity at, or near, the Phe_{105}-Met_{106} bond of κ-casein, a 70% higher clotting activity per mol, and only 20–25% of calf chymosin’s proteolytic activity after cheesemaking, resulting in a 7-fold higher ratio of milk clotting to proteolytic activity (Kappeler et al., 2006). Therefore, significantly less camel chymosin is needed to achieve the same amount of milk clotting. In this study, we minimized the amount of rennet added and used camel chymosin to help reduce proteolytic breakdown in cheese during ripening due to the activity of residual rennet.

Studies on cheese made from concentrated milk have also explored reducing the rennet level to help control proteolysis during cheese storage (Creamer et al., 1987; Spangler et al., 1990). High rennet levels have been associated with bitterness, while very low levels may result in slower proteolysis. Previous studies on calf chymosin have found conflicting results for the relationship between amount of rennet added to the cheesemilk and the concentration of residual rennet in curd. Some have found a linear correlation (Visser, 1977; Zoon et al., 1994), while others have found that up to 5-fold change in the calf rennet concentration added to cheesemilk had no effect on the residual rennet levels obtained in the final cheese (Bansal et al., 2007).

Additionally, previous studies have shown that the application of high-pressure processing (HPP) of > 500 MPa to cheese impacts proteolysis, acid development, and protein structure (Martínez-Rodríguez et al., 2012). The impact of HPP on chymosin activity may depend on the cheese composition and rennet type. We believe that using UF milk, maintaining a high draining pH to retain more INSOL Ca, and using a less proteolytic rennet should slow the breakdown of Cheddar cheese during aging. We investigated if HPP and reducing the level of added rennet could increase intact CN levels in our experimental Cheddar cheeses and thus impact process cheese properties. Our objectives were to evaluate the impact of rennet level and HPP treatment on the functional properties of Cheddar cheese during storage and then to investigate the properties of process cheese made from these Cheddar samples during storage.

**MATERIALS AND METHODS**

**UF of Milk**

Raw whole milk was obtained from the University of Wisconsin-Madison dairy plant 2 d before cheesemaking. Low-concentration-factor UF was performed on whole milk to approximately 17.5 ± 0.5% TS (4.5 ± 0.13% CN). The low-concentration-factor UF was performed at less than 7°C, by recirculation through a UF unit (modified APV North America Inc., Tonawanda, NY) fitted with 6 spiral-wound, polyethersulfone membranes (model ST-3B-4338, Synder Filtration, Vacaville, CA). The elements were housed within 3 parallel vessels, each containing 2 elements in series. Each membrane had a molecular weight cut-off of 10,000 Da and the total membrane area was 43.2 m². The feed spacer
was 31 mm in thickness. The UF retentate was then stored overnight at 4°C and blended to the specified CN content (4.5%) and CN-to-fat ratio the following morning to give standardized cheese milks.

**Cheese Manufacture**

**Experimental cheese treatments.** A licensed Wisconsin cheese maker manufactured 4 independent batches of milled-curd Cheddar cheese at the University of Wisconsin-Madison Dairy Plant. The blended milk was pasteurized at 74.4°C for 19 s, then cooled to set temperatures of 32, 35, and 37.8°C for cheese made with low rennet, 25% less rennet and 50% less rennet, respectively. The different set temperatures were used to be able to use less rennet and still clot the milk in < 30 min. The milk was inoculated with a thermophilic/mesophilic culture containing *Lactococcus lactis* ssp. *lactis* and * cremoris* and *Streptococcus thermophilus* (CH205: DSM Foods, Waukesha, WI) at the rate 26 g per 250 kg of milk. Calcium chloride (32% wt/wt) was added to the milk at a rate of 48 mL per 250 kg of milk. Camel chymosin (Chymax M: Chr. Hansen, Milwaukee, WI) was added at the rate of 38 IMCU/mL, 28 IMCU/mL, and 20 IMCU/mL per 250 kg of milk for the low, low with 25% less rennet, and low with 50% less rennet cheeses, respectively. The coagulum was cut with 12.7 mm knives, and the curd was given a 21-min healing time before cooking. The temperatures of the curd-whey mixtures were raised to 38.9°C over a period of 30 min, before draining the whey when the curd had a pH value of approximately 6.45. Curd slabs were cheddared, stacked 2 high, and milled at pH ~5.86. Curd was salted at the rate of 700 g per 22.7 kg of curd at around 45 min after milling. Curd was packed in 11 kg Wilson-style hoops and pressed at 414 kPa at ambient temperature for 3 h until the pH of the cheese reached about 5.00. The cheese blocks were vacuum packaged and aged at 4°C for up to 240 d.

**High Pressure Processing of Cheddar Cheese**

Experimental Cheddar cheeses were HPP treated 4 d after manufacture. A commercial high-pressure unit (Avure Ultra 215 L, Avure Technologies Inc., WA, USA) located at American Pasteurization Company (Milwaukee, WI) was used to pressure treat the cheese blocks and this unit was able to reach pressures of up to 600 MPa. Water was used as the pressure transfer medium. The temperature of the water in the HPP unit was between 7 to 11°C. The 11 kg cheese blocks were loaded into the unit, and the pressure was increased to 600 MPa over 2–2.5 min. The pressure was maintained for 3 min, then decreased back to atmospheric pressure. An untreated block was used as a control for each of the rennet treatments.

**Process Cheese Manufacture**

Process cheese was made from the experimental Cheddar cheese bases after 14, 60, 120, 180, and 240 d of ripening at 4°C. The Cheddar base was shredded in an Urschel mill (Urchel CC-DL, Urschel Laboratories, Inc., Chesterton, IN). The shreds were combined with water for a 38% moisture target and 2.5% (wt/wt) tri-sodium citrate (TSC) was added. The ingredients were combined in a low shear, twin screw process cheese cooker (Loos model, Loos Machine and Automation; Colby, WI), pre-blended for 1 min, heated to 77°C using indirect steam and held at that temperature for 30 s. The molten process cheese was stretched with a metal spatula ~0.5 m from the cooker to visually (subjectively) determine approximate intact CN characteristics. The process cheese was then discharged into lined 5 lb loaf boxes. Process cheeses were held at 4°C for 1 wk before analysis.
**Compositional Analyses**

The cheese milk was analyzed for fat by the Mojonier method (AOAC, 2000), protein by the total percentage N × 6.38 from the Kjeldahl method (AOAC, 2000), CN (AOAC, 2000), lactose (high pressure ion-exchange chromatography; HPIC; Dionex ICS-5000 RFIC-EGTM Dual System, Thermo Fisher Scientific Inc., Waltham, MA; Möller et al., 2012), TS (Green and Park, 1980), total calcium using inductively coupled argon plasma emission spectroscopy (ICP—OES; Govindasamy et al., 2007), and INSOL calcium by the acid-base titration method (Lucey et al., 1993; Hassan et al., 2004). Cheesemilk was used to generate a rennet whey (Lucey et al., 1993), which was then analyzed for soluble calcium by ICP (AOAC, 2000). The proportion of INSOL Ca was then determined by subtracting soluble Ca from the total Ca content of milk (Hassan et al., 2004).

For cheeses, a slab 2.5 cm thick was cut off the block, and the outer edges were discarded. This slab was further sampled for each analysis. The composition of the ground cheese samples were analyzed at 4 d for moisture (Marshall, 1992), fat (AOAC, 2000), pH using a spear-tip pH electrode (AB15; Fisher Scientific, Hampton, NH), protein (Kjeldahl method; AOAC, 2000), salt (chloride electrode method; Johnson and Olson, 1985), lactose/galactose and lactic acid (HPIC; Möller et al., 2012), and total Ca and Na (ICP-OES; Govindasamy-Lucey et al., 2007). The degree of proteolysis was determined by preparing a pH 4.6 soluble extract (Kuchroo and Fox, 1982), then measuring the amount of total N in these cheese extracts using the Kjeldahl method (AOAC, 2000), which was expressed as a percentage of the total N in the cheese. The buffering capacity of the natural cheese was determined using the method described by Lucey et al. (1993), and Hassan et al. (2004), which was then used to calculate the amount of INSOL Ca in cheese as a percentage of total Ca.

Process cheeses were analyzed 1 wk after manufacture for moisture (Marshall, 1992), fat (AOAC, 2000), pH using a spear-tip pH electrode (AB15; Fisher Scientific, Hampton, NH), protein (Kjeldahl method; AOAC, 2000), and total Ca and Na (ICP-OES: AOAC, 2000).

**Rheological Measurements**

Dynamic small amplitude oscillatory rheology was performed on cheese to determine the melting characteristics. An Anton Paar 302 rheometer (Ashland, VA) with a 50-mm serrated parallel plate geometry was used. Cheese samples, 50 mm in diameter and 3 mm thickness, were prepared and heated from 5 to 95°C at a heating rate of 1°C/min. A frequency of 0.08 Hz and a strain of 0.5% (Govindasamy-Lucey et al., 2005) were applied to measure the storage modulus (\(G'\)), loss modulus (\(G''\)), and loss tangent (\(\tan \delta\)), which is the ratio between the viscous properties and the elastic properties of the material (\(\tan \delta = G''/G'\)). The cross-over temperature (an indicator of melt temperature), maximum loss tangent (\(\tan \delta_{\text{max}}\)), and the temperature at which the \(\tan \delta_{\text{max}}\) was reached were measured. The elastic component of the storage modulus indicates the amount of energy stored, and the viscous component of the loss modulus indicates the amount of energy dissipated through generated heat (Norton et al., 2011).

**Microbiological Analyses**

Starter and non-starter lactic acid bacteria (NSLAB) numbers were measured using deMan, Rogosa, and Sharpe (MRS) agar, and Rogosa SL media, respectively, incubated at 32°C for 48 h under anaerobic conditions (Frank and Yousef, 2004).

**Texture Profile Analysis**

Texture profile analysis (TPA) was measured using a TA.XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY). Samples were prepared with a cork borer with a diameter of 16 mm and a height of 17.5 mm. For the TPA test, cheeses were compressed twice by 30% using a 50-mm aluminum cylinder test probe with a cross-head speed of 0.8 mm/s.

**Residual Rennet Enzyme Analysis**

The residual chymosin activity in Cheddar cheese was measured as described in previous studies (Hurley et al., 1999). Finely grated Cheddar cheese samples (50 mg) were weighed into a 1.5 mL microcentrifuge tube, to which 1 mL of 0.1 M TSC was added. The tubes were incubated in a water bath at 37°C and agitated for 15 s at 5 min intervals until the protein was dissolved. The samples were centrifuged at 1000 × g (Micro Cen- taur, Sanyo, Gallenkamp, UK) for 1 min to separate the fat. A 70 μL sample of the subnatant aqueous layer was used for the analysis of chymosin activity. The subnatant aqueous sample was added to 30 μL of a 1 mg/ mL aqueous solution of a synthetic hepapeptide substrate (Pro-Thr-Glu-[NO2-Phe]-Arg-Leu; Bachem 111 Feichemikalien AG, Switzerland) and 200 μL of 100 mM sodium formate buffer, pH 3.2 (BDH, Poole, Dorset, UK). The mixture was incubated at 37°C for 24 h and the reaction was stopped by heating at 70°C for 10 min. Samples were centrifuged at 16,000 × g for 10 min and the supernatant was analyzed by high perfor-
performance liquid chromatography for the chymosin-induced hydrolysis product ([NO₂-Phe]-Arg-Leu) (Hurley et al., 1999).

**Sensory Analysis of Process Cheese**

A quantitative descriptive analysis of process cheese texture and flavors were evaluated by sensory panelists (n ≥ 9) who had a minimum of 40 h of training according to the method described by Meilgaard et al. (1999). The cheese was evaluated in the form of uniform cubes at 11°C for firmness, cohesiveness, chewiness, adhesiveness, particle size, sweetness, saltiness, bitterness, acidity, milkfat, cooked, buttery, brothy, sourness, sulfur, rancid, cardboardy, processed, astrignency, and burn on a 15-point scale 0 being absence of the characteristic and 15 being overwhelming presence of the characteristic as described by Reale et al. (2020). Processed flavor was evaluated as aromatics associated with process cheese, using Velveeta as a reference. These analyses were performed only on the process cheese that was made from the 180- and 240-d aged natural Cheddar cheese. No sensory analysis was performed on the natural Cheddar cheese.

**Urea-PAGE of Cheddar Cheese**

Urea-PAGE was carried out to monitor the breakdown of β- and αs1-CN. Urea-PAGE gels were prepared according to the method reported by Andrews (1983), as modified by Shalabi and Fox (1987). The 10% (wt/wt) separating gels consisted of 40% (wt/vol) acrylamide solution, N,N-methylene bisacrylamide, and a Tris-HCl buffer (12.5% acrylamide (wt/wt), 4% crosslinking agent (wt/wt), pH 8.9). Stacking gels consisted of 40% (wt/vol) acrylamide solution, N,N-methylene bisacrylamide, and a Tris-HCl buffer (7.5 g Tris + 260 g urea + 4 mL concentrated HCl, dissolved in water and diluted to 1 L, pH 7.6). Cheese samples were standardized to a protein content of 20 μg and dissolved in the sample buffer (0.75 g Tris + 48 g urea + 0.4 mL HCl + 0.2 mL 2-mercaptoethanol + 2 drops 0.25% (wt/vol) bromophenol blue) at 55°C according to the method described by Shalabi and Fox (1987). The samples were then loaded onto the gels held in a Hoefer SE600 Standard Dual Cooled Gel Electrophoresis Unit (Holliston, MA) and allowed to run at a constant voltage of 300 V to completion. Gels were stained with 0.04% (wt/vol) Coomassie Brilliant Blue G250 (BioSafe Coomassie Stain, Bio-Rad Laboratories Inc., Hercules, CA) according to the method by Blakesley and Boezi (1977) and destained with deionized water. Gels were imaged and then analyzed with densitometric analysis software (Gel Analyzer 2010a, developed by Istvan Lazar, Hungary).

**Experimental Design and Statistical Analysis**

Four replicate cheesemaking trials were carried out over a period of 5 mo. In each trial, variable amounts of rennet were added to each of the 3 vats containing the same milk (i.e., 38 IMCU/mL rennet/250 kg milk or control, 28 IMCU/mL rennet/250 kg milk, and 20 IMCU/mL rennet/250 kg milk) used to make Cheddar cheese. These cheeses were split into 2 groups after manufacture, one of which received no pressure treatment as a control, while the others underwent HPP treatment of 600 MPa for 3 min, which was done 4 d after cheese manufacture. A 6 × 4 block design, which incorporated all 6 treatments and all 4 replicate trials, was used for analysis of the response variables related to cheese composition. Analysis of variance was performed using SAS (version 9.4; SAS Institute, Cary, NC). The 3 different levels of rennet added to the cheesemilk and the 2 HPP treatments were analyzed as discontinuous variables where the 4 cheesemaking trials were blocked. Duncan’s multiple comparison test was carried out to evaluate differences in treatment means. A significance level of P < 0.05 was used.

A split-split-plot design was used to monitor the effects of both treatments (rennet level and HPP) and ripening time and their interactions on pH, LT max, LT max temperature, crossover point temperature, hardness, proteolysis, insoluble calcium, and sensory attributes during ripening. For the whole- and sub-plot factors, both the treatments were analyzed as a discontinuous variable and the cheesemaking trial was blocked. For the sub-subplot factor analysis, age was treated as a continuous variable. Analysis of variance for the split-split-plot design was carried out with SAS. When significant differences (P < 0.05) were found, the means of the different treatments were analyzed using Duncan’s multiple-comparison test.

**RESULTS AND DISCUSSION**

**Composition of Cheeses**

There were no significant differences in moisture, protein, salt, total Ca, percent fat in dry matter (FDM), and percent of moisture in nonfat substance (MNFS) from the rennet level or HPP treatments (Table 1). The industry standard-make of Cheddar had a slightly lower moisture and Ca contents, and slightly higher fat and protein contents (Table 1). The lower Ca content was expected due to its lower draining pH compared with the experimental cheeses.
HPP treatment resulted in a significant reduction in starter numbers (from 0.3 to 3.8 log reduction at 4 d and 6 mo, respectively) and starter counts also decreased significantly during storage (Table 2), especially for the HPP-treated cheeses. It is well documented that HPP-treatment significantly decreases the number of viable starters (Wick et al., 2004; Rynne et al., 2008; Ozturk et al., 2013a, 2013b, 2015; Martinez-Rodriguez et al., 2012). The high pressure causes structural and functional damage to vegetative cells, leading to cell injury or death. These potential changes include cell membrane disruption or increased permeability, ribosomal destruction, collapse of intracellular vacuoles, denaturation of membrane-bound proteins, damage to the proton efflux system, and inactivation of key enzymes involved in DNA replication and transcription (Martinez-Rodriguez et al., 2012). It is likely that the HPP treatment inactivated some of the starter bacteria and damaged more cells that died off during the remainder of the ripening period. The NSLAB numbers were very low initially in all experimental cheese (<10^2 cfu/ml) but increased with storage (results not shown).

The lactic acid levels in all experimental Cheddar cheeses increased throughout the ripening period (Table 3). The lactic acid values in experimental Cheddar cheeses were similar at 4 d of ripening. When Cheddar cheeses had ripened ≥ 60 d, the HPP-treated cheeses had lower lactic acid values (Table 2), likely due to the lower levels (or activities) of starter bacteria that were able to convert lactose into lactic acid.

The pH of the experimental cheeses (Table 3) was higher than typical Cheddar cheese due to the increased buffering capacity in the cheese, a result likely due to the use of UF milk (Green et al., 1981) and the high draining pH value (Johnson and Lucey, 2006). The rennet level had no significant effect on the pH of the cheeses (Table 2). The HPP treatment increased the initial pH of the experimental cheese for all rennet levels (Table 2); in agreement with numerous previous studies in Cheddar (Ozturk et al., 2013a; Rynne et al., 2008), Gouda (Messens et al., 1999; Messens et al., 2000), Mozzarella (Johnston and Darcy, 2000; Sheehan et al., 2005), goat milk (Saldo et al., 2002), and ewe milk cheeses (Juan et al., 2007). A combination of rennet level and HPP treatments did not significantly affect the pH value (Table 3). Several possible mechanisms could explain the (small) initial pH increase in cheese after HPP treatment. Solubilization of INSOL calcium phosphate (with the release of phosphate ions) can occur while the pressure is being applied during HPP (Huppertz et al., 2004a; Ozturk et al., 2013a), and/or the pressure may inactivate the starter bacteria or their glycolytic enzymes before the complete fermentation of lactose to lactic acid (Rynne et al., 2008; Malone et al., 2003; Ozturk et al., 2013a). The pH of the non-pressurized cheese increased over the first 120 d of ripening, then fluctuated slightly for the remaining ripening period.
The pH of HPP-treated cheese increased over the first 60 d of ripening, then remained stable and mostly consistent for the remaining ripening period. The increase in pH of the Cheddar cheese in the first few weeks of ripening can be attributed to the solubilization of INSOL calcium phosphate from the casein micelle matrix as it moves toward a pseudo-equilibrium between soluble and insoluble states (Johnson and Lucey, 2006). The mean composition of process cheese was 37.7% moisture, 31.3% fat, 24.1% protein and 762 mg Ca/100 g cheese. There was no difference in the composition of process cheese made from any of the experimental treatments (results not shown). The pH of the process cheese made from the experimental treatments were similar and typical for process cheeses (pH ~5.8–5.9). The trend of a slightly higher pH in the HPP-treated Cheddar cheese was also observed in the process cheese (results not shown). This was likely due to the increased buffering capacity of the natural Cheddar cheese with high Ca and phosphate contents (Upreti and Metzger, 2007) and has been found in other studies (Kapoor et al., 2007; Acharya and Mistry, 2005).

### Insoluble Calcium

The amount of INSOL Ca (as a percentage of total calcium) in all the experimental Cheddar cheeses (Figure 1) remained high during the ripening period due to the high Ca and phosphate contents of the natural Cheddar cheese.

### Table 2. Probabilities and R² values for starter, lactic acid, pH, insoluble Ca, rennet activity, pH 4.6 soluble N, LT₅₀, crossover temperature, and hardness of natural Cheddar cheeses manufactured with various rennet levels and HPP-treatments

<table>
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<tr>
<th>Factors¹</th>
<th>df²</th>
<th>Starter</th>
<th>Lactic acid</th>
<th>pH</th>
<th>Insoluble Ca</th>
<th>pH 4.6 soluble N</th>
<th>LT₅₀</th>
<th>Crossover temperature</th>
<th>Hardness</th>
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<td>Day of cheese manufacture (D)</td>
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<td>&lt;0.05</td>
<td>0.06</td>
<td>0.0004</td>
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<td>0.44</td>
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<td>&lt;0.05</td>
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<td>Age (A)</td>
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<td>&lt;0.0001</td>
<td>0.09</td>
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<td>(RL × A)</td>
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<td>0.74</td>
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<tr>
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<td>0.90</td>
<td>0.99</td>
<td>0.99</td>
<td>0.79</td>
<td>0.99</td>
<td>0.73</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>R²</td>
<td>0.88</td>
<td>0.80</td>
<td>0.63</td>
<td>0.78</td>
<td>0.98</td>
<td>0.87</td>
<td>0.82</td>
<td>0.70</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Split-split-plot design with the three treatments (control (38 IMCU/mL rennet/250 kg milk), 25% reduced, and 50% reduced rennet levels) were analyzed as a discontinuous variable and day of cheese manufacture was blocked. Subplot included the effect of applying HPP-treatment after manufacture and the interactions as variables. The sub-subplot included the effect of aging of cheese and the interactions as variables. The R² value given was for the whole model.

2Degrees of freedom differed for rennet activity, as it was only tested at one time point, 4 d after manufacture.

3Not determined.

### Table 3. pH values and lactic acid content (%) of natural Cheddar cheese manufactured with various rennet levels and HPP-treatments over ripening period

<table>
<thead>
<tr>
<th>pH and Lactic Acid Values of Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Rennet Level</td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Non-HPP HPP</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>Non-HPP HPP</td>
</tr>
<tr>
<td>HPP</td>
</tr>
<tr>
<td>Total Lactic acid (%)</td>
</tr>
<tr>
<td>Non-HPP HPP</td>
</tr>
<tr>
<td>HPP</td>
</tr>
</tbody>
</table>

Means within the same row not sharing a common superscript differ (P < 0.05).

Values represent the means of 4 replicates for each treatment.
to its high draining pH used for cheesemaking, the use of concentrated milk, which provided more buffering capacity to the curd, as well as a high cheese pH (Table 3) (Lucey et al., 2003; Ozturk et al., 2015). Calcium is more readily solubilized when acid development occurs in the vat (as milk) during cheesemaking (Lucey and Fox, 1993); thus, draining the whey at a higher curd pH reduced the amount of Ca solubilized and much of remaining Ca in cheese was predominantly in the INSOL form (Johnson and Lucey, 2006). The concentration of milk by UF increases the buffering capacity of the milk, which increases the cheese pH (Green et al., 1981).

For the experimental treatments, the increase in cheese pH from 4 to 120 d (Table 3) helped to reduce the driving force for further solubilization of INSOL calcium phosphate crosslinking and retain higher INSOL Ca levels in the cheese during ripening. The high pH level in the HPP-treated cheeses (Table 3) also probably helped to maintain high levels of INSOL calcium phosphate during ripening in the HPP-treated cheeses. Ozturk et al. (2013a; 2013b; 2015) observed that HPP did not affect the INSOL Ca content in low sodium, reduced fat Cheddar cheese or Cheddar cheese made with UF milk during ripening. In our study, the HPP-treated cheeses maintained slightly higher levels of INSOL Ca throughout the 240-d ripening period than cheeses not HPP-treated, which was consistent with the higher pH in the HPP-treated cheeses, which hardly changed throughout ripening. The INSOL Ca levels in all samples exhibited a small decrease between 4 and 120 d of ripening, then remained constant for the remaining ripening period (Figure 1); in agreement with previous reports on Cheddar cheese (Hassan et al., 2004). Rennet levels significantly impacted INSOL Ca levels (Table 2), at 4 d of ripening, the cheeses made with the control level of rennet had a lower level of INSOL Ca (Figure 1), possibly due to the different set temperatures used. A combination of rennet level and HPP treatments had no significant effect on levels of INSOL Ca (Table 2).

**Proteolysis**

The residual rennet activity in the experimental Cheddar cheeses was not significantly (Table 2) affected by reducing the amount of rennet added to the cheese milk (Figure 2). Bansal et al. (2007) also observed no difference in the amount of rennet retained in cheese with an increase in the concentration of rennet added to the cheesemilk. These results suggest that the amount of (camel) rennet bound to CNs was almost constant, and the remaining (unbound) rennet was lost in the whey (Bansal et al., 2007). Creamer et al. (1987) observed a linear decrease in residual rennet as the amount of calf rennet added to the Cheddar cheesemilk was decreased.

The difference in rennet retention results may be due to the higher concentration (5-fold) of milk in the study conducted by Creamer et al. (1987) compared with the 1.8 × concentrated cheese milk in this study. The pH of milk at renneting, or the rennet types, may also impact rennet retention in cheese curd.

Residual rennet activity was significantly decreased by HPP treatment (Figure 2; Table 2). It is well documented in many cheese varieties that HPP treatment ≥ 400 MPa decreases rennet activity (Saldo et al., 2002; Huppertz et al., 2004b; Juan et al., 2007). Saldo et al. (2002) reported that the residual rennet activity was reduced by half in Garrotxa cheese after an HPP treatment of 400 MPa for 5 min on the first d after cheese manufacture. Huppertz et al. (2004b) observed rennet activity to be decreased to < 10% at pressures of 600 and 800 MPa for ≥ 15 min. The decrease in residual rennet activity in this study was 21–31%, likely lower than the previous studies due to the shorter pressure holding time. A combination of rennet level and HPP treatments had no significant effect on residual rennet activity (Table 2).

The proteolytic breakdown of intact CN, as measured by the level of pH 4.6 soluble N as a percentage of total N, was significantly reduced during ripening by using camel chymosin in the experimental cheeses, compared with the calf chymosin used for the standard industry-make cheese (Figure 3). The use of camel chymosin reduces the breakdown of intact CN due to the lower proteolytic activity of camel chymosin when compared with the traditionally used calf chymosin (Kappeler et

![Figure 1. Insoluble Ca as a percentage of total Ca values for control (●), 25% reduced (▲), and 50% reduced (■) rennet levels, non-treated and HPP-treated (closed symbols denote non-treated, open symbols denote HPP-treated cheese) at 4, 60, 120, 180, and 240 d of ripening at 4°C. Vertical bars represent standard deviations.](image-url)
Bansal et al. (2009) found that between 15 and 180 d of ripening there was an increase in pH 4.6 soluble N as a percent of total N from about 8 to 19%, and 5 to 15%, in Cheddar cheese made with calf and camel chymosin, respectively. These results were similar to the trends we observed.

Changing the level of rennet added to the cheesemilk and HPP processing had a small (Figure 3) but significant (Table 2) effect on pH 4.6 soluble N as a percent of total N. Previous studies have shown high HPP treatments (e.g., 600 MPa) could partly decrease the activity of the residual rennet and bacterial proteinases, which both play a role in cheese proteolysis (Ozturk et al., 2015; Wick et al., 2004). By 240 d of ripening, pH 4.6 soluble N of the cheese made with 50% reduced rennet and HPP-treated was significantly lower than the other cheeses (Figure 3). The interaction between rennet level and HPP treatment had no significant effect on levels of pH 4.6 soluble N breakdown (Table 2). Figure 3 indicated that the use of UF milk and camel chymosin had a greater influence on slowing the release of pH 4.6 soluble N components, than reducing the amount of camel rennet added or applying a HPP treatment of 600 MPa for 3 min.

The urea-PAGE gel (Figure 4) of experimental cheeses after 14 d of ripening showed very minimal protein breakdown and no significant differences between treatments. However, at 240 d of ripening, the HPP-treated samples exhibited slightly more β-CN breakdown than the unpressurized samples and less αs1-CN breakdown (Figure 4). The lower extent of αs1-CN breakdown in the HPP-treated cheeses agreed with the observed decrease in residual rennet activity in the HPP-treated cheeses (Figure 2). The increase in β-CN breakdown in HPP-treated cheeses has not been observed in previous studies. Other studies have reported that HPP had little effect on β-CN breakdown (Ozturk et al., 2015) or plasmin activity in cheese (Rynne et al., 2008; O’Reilly et al., 2002; Huppertz et al., 2004b). One possible explanation is that HPP treatment of cheese inactivated a plasmin inhibitor. Another possibility is related to the increase in cheese pH caused by HPP treatment (Table 3), a higher pH could elevate plasmin activity since this enzyme has an alkaline pH optimum.

The impact of differences in intact CN levels during aging from 14 to 240 d was visually apparent when the molten process cheese was manually stretched immediately after cooking to 77°C (Figure 5). If a thick strand of process cheese was observed when stretched this indicated that the natural cheese, from which the process cheese was manufactured, had a high level of intact CN. All process cheeses made from cheeses ripened for 14 d had thick strands (Figure 5a). As the CNs in the natural cheese were broken down by proteolysis (by 240 d), the hot strands of process cheese became thinner and eventually broke shortly after the stretching began, this was very obvious in the process cheese made from the industry standard make Cheddar sample (Figure 5d). The high level of INSOL Ca early in the ripening phase may have contributed to the thick strands of molten process cheese at 14 d, but it was not the critical factor, as a large decrease in the strand properties was seen over the ripening period with little change in INSOL.
Ca after 60 d of ripening. The experimental cheeses aged for 240 d had similar hot stretching properties (Figures 5b, c) and they were superior to the aged industry standard-make process cheese (Figure 5d).

**Rheology**

The number, strength, and type of bonds between CNs in the cheese matrix determine the rheological behavior of cheese (Lucey et al., 2003). The LT\textsubscript{max} value is used as an indication of cheese meltability (Lucey et al., 2003). The LT\textsubscript{max} values of the experimental Cheddar cheese samples increased during ripening (Figure 6a), which was in agreement with their decrease in intact CN (Figure 3). Initially, HPP-treated Cheddar cheeses had higher LT\textsubscript{max} values than non-pressurized cheeses but from > 100 d onwards the HPP-treated cheeses had lower LT\textsubscript{max} values (Figure 6a). LT\textsubscript{max} values were significantly impacted by HPP treatment (Table 2). The higher initial LT\textsubscript{max} values in HPP treated Cheddar cheese could be due to the solubilization of INSOL calcium phosphate crosslinking by the pressure treatment. The higher residual activity in the non-pressurized Cheddar cheeses compared with HPP-treated cheeses

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**Figure 4.** Urea-PAGE electrophoretograms showing the breakdown of caseins in control, 25% reduced, and 50% reduced rennet levels, non-treated (C) and HPP-treated Cheddar cheeses at 14 and 240 d of ripening at 4°C.
(Figure 2) likely helped to increase the meltability of the non-pressurized cheeses during ripening. This was similar to the trends reported by Öztürk et al. (2013b). A combination of rennet level and HPP treatment significantly impacted the LT\textsubscript{max} values (Table 2). The HPP treatment did significantly increase the pH of the treated cheeses (Table 3), which could restrict the melt, due to 2 possible influences: a decrease in the ratio of soluble-to-INSOL calcium, or a decrease of the degree of para-casein hydration (Guinee, 2002). Guinee et al. (2002) reported that an increase in the pH in Mozzarella cheese resulted in significantly lower stretchability and flowability values over the entire ripening period. The LT\textsubscript{max} temperature slightly decreased over the ripening period (results not shown), in agreement with the intact CN breakdown results (Figure 3). The breakdown of intact CN decreases the number of CN-CN interactions, so the matrix requires less heat (thermal) energy to reach the LT\textsubscript{max} (Lucey et al., 2003). However, the temperature at which the LT\textsubscript{max} was reached was not significantly impacted by the rennet levels or HPP treatments (results not shown).

The temperature at which the $G'$ and $G''$ moduli crossover during a rheology heating test is referred to as the crossover point, which is an indication of the melt temperature. No significant differences in the crossover point of Cheddar cheeses were observed between treatments (Table 2), although it did slightly decrease during the ripening period (results not shown). This decrease was likely due to an increase in electrostatic repulsion from the reduction in INSOL calcium phosphate cross-linking, making it easier for the CN-CN bonds to relax and flow over each other during heating (Lucey et al., 2003).

Following similar trends in the Cheddar cheese, process cheese also became more meltable when prepared from older Cheddar cheese, as indicated by an increase in the LT\textsubscript{max} value (Figure 6b). The HPP-treatment of the Cheddar cheese significantly lowered the LT\textsubscript{max} value of the process cheese, thus, restricting its meltability, and this impact was observed for process cheeses made from all ages of natural Cheddar cheese (Figure 6b). Greater dispersion of natural cheese results in less meltable process cheeses; this dispersion is usually achieved by the use of higher levels of emulsifying salts (Lucey et al., 2011). One possibility is that the HPP process causes a disruption of the CNs and fat globules, and that the disruption of these interactions by HPP promotes greater dispersion during process cheesemaking.

Within a HPP treatment, there was no significant differences in the LT\textsubscript{max} values for the process cheeses with the various rennet levels (Figure 6b). This trend was similar to the residual rennet activity results, where HPP reduced residual activity but rennet level had no impact (Figure 2). The LT\textsubscript{max} values were similar for non-pressurized natural Cheddar cheeses (Figure 6a) and their respective process cheeses (Figure 6b), although the process cheeses had slightly higher LT\textsubscript{max} values.

The $G'$ value at a measuring temperature of 70°C was used to explore the residual stiffness of cheese after heating/melting. The $G'$ values at 70°C for Cheddar cheese decreased significantly during ripening (Figure 7a); similar trends have been observed previously (Lucey et al., 2005). Similar to the trends for the LT\textsubscript{max} values (Figure 6b), HPP-treatment of the Cheddar cheese significantly increased the $G'$ values at 70°C of the resultant process cheese (Figure 7b), thus, indicat-
ing higher residual stiffness after melting. There were little differences in the $G'$ values at 70°C for process cheeses made with different rennet levels (Figure 7b). The $G'$ values at 70°C for process cheese decreased with an increase in the age of the natural cheese.

**Texture**

The hardness of the experimental Cheddar cheese (Figure 8a) exhibited an initial decrease from 4 to 60 d, which agreed with the initial decrease in INSOL Ca content (Figure 1) and is typical in the first 60 d of ripening (Lucey et al., 2003; O’Mahony et al., 2005). Hardness remained consistent for the remaining ripening period in all experimental treatments, also in agreement with the lack of further change in the levels of INSOL Ca. Hardness was significantly impacted by rennet level but not by HPP treatment (Table 2). A combination of rennet level and HPP treatments had no significant effect on the hardness (Table 2). In the natural Cheddar cheeses throughout the ripening period, the control rennet level samples had higher hardness values than the cheeses made with 25 and 50% reduced rennet levels. Creamer et al. (1987) observed a different trend, that a decrease in the amount of rennet added to the cheese increased the hardness. Possible explanations for our trends could include our control cheese had slightly lower moisture and higher salt-in-moisture levels than the reduced rennet samples (Table 1). HPP treatment had no significant effect on the hardness of the cheese.

After the Cheddar cheese had been ripened 14 d, the hardness of the resultant process cheese (Figure
8b) with the control rennet level was higher than those made from the reduced rennet natural cheeses. For process cheeses, at some of the time points the hardness of the HPP treated cheeses were slightly higher than the corresponding non-pressurized cheeses.

**Sensory**

The sensory textural and flavor attributes of the process cheeses made from Cheddar cheese is shown in Table 4. Descriptive sensory was only performed on the process cheese made from Cheddar cheese ripened for 180 and 240 d to determine the texture and flavor attributes that may be present after the natural Cheddar cheese base would have been exported and made into process cheese. The sensory attributes of the process cheese were not impacted by rennet level or HPP treatment. Panelists detected no significant difference in any textural attributes, such as firmness, cohesiveness, chewiness, adhesiveness, and particle size between treatments. It was noticeable that key textural attributes in our process cheeses like firmness, cohesiveness and chewiness, as well as flavor attributes like buttery and processed, did not change in cheeses that had been aged for 180 or 240 d. There was a decrease in some flavors of our process cheese with the age of Cheddar cheese, such as, attributes like milkfat and cooked notes (Table 4).

**CONCLUSIONS**

High pressure treatment of Cheddar cheese significantly impacted starter/nonstarter numbers, pH development, INSOL calcium levels, and proteolysis but did not significantly impact hardness nor the melting temperature. HPP reduced starter numbers and reduced residual rennet activity, which contributed to lower proteolysis in the Cheddar cheese. We did notice that the rate of breakdown of β-CN was higher in HPP treated Cheddar cheeses, which was unexpected, possibly due to the slight increase in pH after HPP treatment that would favor plasmin activity. HPP resulted in an initial reduction in the INSOL calcium content (reduced protein crosslinking) due to the pressure treatment, but over ripening this negative impact on textural properties like hardness might have been compensated by the reduced proteolysis in HPP-treated Cheddar cheeses. We also prepared a Cheddar cheese with more standard-make procedures, but all our experimental Cheddar cheeses had much higher levels of intact CN during ripening, than the industry make cheeses, likely due to their use of a less proteolytic rennet, higher draining pH values, and higher levels of total and INSOL calcium. Reducing the amount of rennet added to cheese milk did not significantly impact residual rennet activity in Cheddar cheese but we observed a slightly slower proteolysis rate with lower amounts of rennet. Rennet level significantly impacted LT_{max} values (an indication of meltability) of Cheddar cheese, with higher LT_{max} values observed during ripening with higher rennet levels (likely due to greater proteolysis). Process cheeses were made from the experimental Cheddar cheeses over ripening for up to 240 d. HPP treatment resulted in process cheese with lower LT_{max} values (lower meltability) and higher storage modulus values at high measuring temperatures (when melted). This indicated that HPP of Cheddar cheese could be used to reduce melt and to prepare process cheese that had more elastic properties when heated. Several cheesemaking and processing conditions were identified that were effective in slowing the

**Figure 8.** Hardness for control (●), 25% reduced (▲), and 50% reduced (■) rennet levels, non-treated and HPP-treated (closed symbols denote non-treated, open symbols denote HPP-treated cheese) natural (a) and process (b) Cheddar cheeses made from natural cheese at 14, 60, 120, 180, and 240 d of ripening at 4°C. Vertical bars represent standard deviations.
breakdown of intact CN and maintaining high levels of insoluble Ca, which could be used by manufacturers as they attempt to export cheese for processing purposes.

ACKNOWLEDGMENTS

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Table 4. Sensory textural and flavor attributes (intensities based on a 0–15 point scale) for process cheese made from ripened Cheddar cheese manufactured with various rennet levels and HPP-treatments over ripening period.

<table>
<thead>
<tr>
<th>Ripening Time (day)</th>
<th>Control Rennet Level</th>
<th>25% Reduced Rennet Level</th>
<th>50% Reduced Rennet Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-HPP</td>
<td>HPP</td>
<td>Non-HPP</td>
</tr>
<tr>
<td>180 d</td>
<td>Firmness</td>
<td>13.0a</td>
<td>13.0a</td>
</tr>
<tr>
<td></td>
<td>Cohesiveness</td>
<td>9.9a</td>
<td>10.1a</td>
</tr>
<tr>
<td></td>
<td>Cheviness</td>
<td>5.9a</td>
<td>5.7a</td>
</tr>
<tr>
<td></td>
<td>Adhesiveness</td>
<td>6.1a</td>
<td>6.0a</td>
</tr>
<tr>
<td></td>
<td>Particle Size</td>
<td>11.5a</td>
<td>11.3a</td>
</tr>
<tr>
<td></td>
<td>Sweet</td>
<td>4.5a</td>
<td>4.4a</td>
</tr>
<tr>
<td></td>
<td>Salt</td>
<td>0.6a</td>
<td>0.5a</td>
</tr>
<tr>
<td></td>
<td>Bitter</td>
<td>0.6a</td>
<td>0.6a</td>
</tr>
<tr>
<td></td>
<td>Milkfat</td>
<td>3.9a</td>
<td>3.7a</td>
</tr>
<tr>
<td></td>
<td>Cooked</td>
<td>3.4a</td>
<td>3.2a</td>
</tr>
<tr>
<td></td>
<td>Buttery</td>
<td>1.3a</td>
<td>1.3a</td>
</tr>
<tr>
<td></td>
<td>Processed</td>
<td>5.9a</td>
<td>5.8a</td>
</tr>
<tr>
<td>240 d</td>
<td>Firmness</td>
<td>13.1a</td>
<td>13.0a</td>
</tr>
<tr>
<td></td>
<td>Cohesiveness</td>
<td>10.0ab</td>
<td>9.9ab</td>
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<tr>
<td></td>
<td>Cheviness</td>
<td>5.7a</td>
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<td>Adhesiveness</td>
<td>5.7a</td>
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<td>Particle Size</td>
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<td></td>
<td>Salt</td>
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<td>5.8a</td>
<td>5.6a</td>
</tr>
</tbody>
</table>

a,bMeans within the same row not sharing a common superscript differ (P < 0.05).
1Values represent the means of 4 replicates for each treatment.
2Ripening time indicates the ripening time of the natural Cheddar; process cheese made from the aged cheese at this stage of ripening.

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