Highlights

- Coagulant type, coagulant inhibitor and ripening temperature influenced casein hydrolysis patterns.
- Level of insoluble calcium decreased significantly during ripening.
- Breakdown of α_{S1}-casein had no pronounced influence on shortness of cheese texture.
- Level of β-casein was negatively associated with the shortness of cheese texture.
- Insoluble calcium levels were negatively associated with the shorter cheese texture.

Microstructure and fracture properties of semi-hard cheese: Differentiating the effects of primary proteolysis and calcium solubilization

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Abstract

The individual roles of hydrolysis of α_{S1}- and β-caseins, and calcium solubilization on the fracture properties of semi-hard cheeses, such as Maasdam and other eye-type cheeses, remain unclear. In this study, the hydrolysis patterns of casein were selectively altered by adding a chymosin inhibitor to the curd/whey mixture during cheese manufacture, by substituting fermentation-produced bovine chymosin (FPBC) with fermentation-produced camel chymosin (FPCC), or by modulating ripening temperature. Moreover, the level of insoluble calcium during ripening was quantified in all cheeses. Addition of a chymosin inhibitor, substitution of FPBC with FPCC, or ripening of cheeses at a consistent low temperature (8 °C) decreased the hydrolysis of α_{S1}-casein by ~95%, ~45%, or ~30%, respectively, after 90 d of ripening, whereas ~35% of β-casein was hydrolysed in that time for all cheeses, except for those ripened at a lower temperature (~17%). The proportion of insoluble calcium as a percentage of total calcium decreased significantly from ~75% to ~60% between 1 and 90 d. The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher fracture stress) in cheeses with lower levels of proteolysis or higher levels of intact caseins, primarily α_{S1}-casein. However, contrary to the expectation that shortness of cheese texture is associated with α_{S1}-casein hydrolysis, fracture strain was significantly positively correlated with the level of intact β-casein and insoluble calcium content, indicating that the cheeses with low levels of intact β-casein or insoluble calcium content were more likely to be shorter in texture (i.e., lower fracture strain). Overall, this study suggests that the fracture properties of cheese can be modified by selective hydrolysis of caseins, altering the level of insoluble calcium or both. Such approaches could be applied to design cheese with specific properties.

Keywords: Cheese; Proteolysis; Insoluble calcium; Fracture properties; Microstructure; Split or crack defect

1. Introduction

Knowledge of fracture properties of cheese is important for understanding breakdown properties of cheese during mastication, in designing cheese texture suitable for size reduction operations (e.g., slicing, dicing or grating), and in understanding the reasons for formation of undesirable texture defects within the cheese matrix, such as slits and cracks (Luyten, 1988).

Development of undesirable slits and cracks within the cheese matrix is an international problem in the manufacture of Swiss, Dutch and related eye-type cheeses, leading to downgrading of the product, resulting in lost revenue to manufacturers (Grappin, Lefier, Dasen, & Pochet, 1993; White, Broadbent, Oberg, & McMahon, 2003; Guggisberg et al., 2015). To date, the exact reasons for development of such defect are not known. However, excessive production of gas, an unsuitable cheese texture or both have been considered as root causes for occurrence of this defect (Daly, McSweeney, & Sheehan, 2010; Rehn et al., 2011). If the cheese texture is short or brittle (i.e., fracturing of cheese matrix at a relatively small deformation), the cheese matrix is no longer able to withstand increased gas pressure during eye-formation or storage, leading to formation of cracks and splits. Although the exact reasons for a cheese to become short or brittle during ripening are not yet fully understood, proteolysis, partial solubilization of colloidal calcium phosphate associated with *para*-casein matrix of the curd during ripening, or both, have been considered as possible reasons (Lucey, Johnson, & Horne, 2003; Daly et al., 2010). However, the role of primary proteolysis and level of insoluble calcium on fracture behaviour of brine-salted semi-hard cheese has not yet been fully elucidated.

From a structural perspective, α_{S1}-casein and β-casein are the two important caseins within the cheese matrix, and these undergo varying degree of hydrolysis during ripening in

different cheese varieties through the action of residual coagulant and plasmin, respectively (Sheehan, O'Sullivan, & Guinee, 2004b; Kelly, O'Flaherty, & Fox, 2006; Lamichhane, Kelly, & Sheehan, 2018b). Studies have suggested that the caseins have different hydrophilic and hydrophobic blocks. For example, α_{S1}-casein has a hydrophilic region between strong hydrophobic regions, whereas the β-casein has a hydrophilic and a hydrophobic region at N- and C-terminal, respectively (Lucey et al., 2003). Thus, these caseins are held together by various molecular forces within the cheese matrix. Moreover, calcium associated with casein enhances the cross-linking of casein within the cheese matrix. Therefore, it is reasonable to assume that both hydrolysis patterns of casein and solubilization of colloidal calcium during ripening alter the casein interactions, which may in turn influence the textural, rheological and fracture behaviour of cheese. A better understanding of the individual contribution of such factors may allow the development of specific strategies to design cheese with specific properties.

Unlike high maximum scald temperatures (~55 °C) in Emmental cheese manufacture, cheese curds are cooked only to ~40 °C during manufacture of most semi-hard cheeses, such as Maasdam and Jarlsberg (Fröhlich-Wyder et al., 2017), which is not sufficient to inactivate or reduce the residual chymosin activity, resulting in extensive breakdown of α_{S1}-casein during ripening (McGoldrick & Fox, 1999). The role of chymosin-mediated proteolysis on texture properties of Cheddar cheese has previously been studied by inhibition of the residual chymosin by the addition of a chymosin inhibitor to the curd-whey mixture (O'Mahony, Lucey, & McSweeney, 2005). However, little is known about the role of chymosin-mediated proteolysis on the fracture behavior of semi-hard Swiss, Dutch and related eye-type cheeses. Some semi-hard eye-type cheeses are ripened in a warm room (~23 °C) for 4-6 weeks for the development of eyes. However, the effect of such elevated ripening temperature on solubilization of calcium and hydrolysis of casein is also not fully understood.

The aim of this study was to decouple and explore the individual role of primary proteolysis (both of α_{S1}- and β-casein) and insoluble calcium on the fracture properties of washed-curd brine-salted semi-hard cheese.

2. Materials and methods

2.1. Milk supply and cheese manufacture

Raw milk was obtained from the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Ireland. Raw milk was first separated into skim milk and cream using bench top centrifugal separator. Using skim milk and cream, cheese milks were standardized to a protein to fat ratio of 1.10:1.00, with an average protein and fat content of 3.52 % (w/w) and 3.21 % (w/w), respectively. The standardized cheese-milks were then pasteurized at 72 °C for 15 sec (MicroThermics, USA) and stored at 4 °C overnight prior to cheese manufacture.

Washed-curd brine-salted semi-hard cheeses were manufactured in triplicate trials over a 3 month period. Standardized and pasteurized cheese milks were placed into jacketed cheese vats (Pierre Guerin Technologies, Niort, France) with each vat containing 11 kg cheese milk, for each replication. Each vat contained automated variable speed cutting and stirring equipment. All cheese milks were inoculated at 32 °C with frozen direct vat inoculation cultures: consisting of (1) R-604 (180 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*; and (2) LH-B02 (9 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactobacillus helveticus*. Propionic acid bacteria were not inoculated into the cheese milks to avoid subsequent eye-formation during ripening of cheese which would not permit measurement of texture parameters.

All cheese milks were pre-acidified to 6.55 using 4% (w/v) lactic acid (Sigma-Aldrich) prior to rennet addition. After 40 min of pre-ripening, the coagulant, fermentation-produced bovine chymosin (FPBC; CHY-MAX Plus, ~200 international milk clotting units (IMCU)/mL; Chr. Hansen Ltd., Cork, Ireland) was added at a level of 2 mL/11 kg cheese milk in 3 out of 4 vats, whereas fermentation-produced camel chymosin (FPCC; CHY-MAX M, ~200 IMCU/mL; Chr. Hansen Ltd., Cork, Ireland), was added at a level of 1.5 mL/11 kg cheese milk in the fourth vat. Coagulants were diluted ~1:10 with deionized water prior addition. The addition rates of both FPBC and FPCC to milk were predetermined through a series of rheological experiments where the levels of the coagulants were adjusted to achieve coagula of similar gel strength (35 Pa) after a set period of ~45 min.

All gels were cut at a constant firmness (G′) value of 35 Pa (as measured using a small-amplitude oscillatory rheometer, AR 2000ex, TA Instruments) and the resultant curd/whey mixture was allowed to heal for 5 min before being stirred continuously for another 10 min. Stirring was then stopped and a portion of whey (0.35 kg/kg cheese milk) was removed. Just after whey removal, in one vat out of four vats, Pepstatin A (synthetic; Enzo life science, Exeter, UK) was added to the curd/whey mixture at a rate of 10.0 μmol/kg cheese milk and evenly distributed by continuous stirring during cooking. Pepstatin A is an inhibitor of aspartic proteases, including chymosin, pepsin, cathepsin D, and renin (Marciniszyn, Hartsuck, & Tang, 1976). After whey removal, reverse osmosis water at ~50 °C (0.25 kg/kg cheese milk) was added to each cheese vat to cook the curd to 37 °C at a rate of 0.2 °C/min with continuous stirring.

Whey was drained when the curd pH reached 6.35, and the curds were collected into moulds and pressed vertically under increasing pressure from 40 to 75 kPa for ~4.5 hours. When the pH of the cheese curds reached ~5.50, the cheese wheels (~600 g each) were transferred to a saturated brine solution (23%, w/w, NaCl, 0.56%, w/w, $CaCl_2$, and pH 5.2) for 7.5 h at 8 °C.

After brining, cheese wheels were vacuum-packed (Falcon 52, Original Henkelman vacuum system, the Netherlands), and transferred to the ripening room. Cheese wheels were ripened at 8 °C for 20 d (pre-ripening), at 23 °C for 28 d (warm-room ripening) or 8 °C for 28 d (without warm room ripening), and finally stored at 4 °C for 42 d. A summary of the experimental plan is shown in Table 1.

2.2. Milk and cheese composition

The composition of raw and pasteurized (72 °C for 15 s) cheese milks were analyzed as described by Lamichhane, Kelly, and Sheehan (2018a). Grated cheese samples were analyzed at 20 d of ripening in duplicate for moisture, fat, protein and salt as described by Hickey et al. (2018b). Cheese pH was measured at 1, 20, 48 and 90 d as described by Sheehan, Fenelon, Wilkinson, and McSweeney (2007).

2.3. Enumeration of starter and nonstarter lactic acid bacteria

Samples were removed from cheese wheels using a cheese trier at 1, 20, 48 and 90 d of ripening. Cheese samples were prepared as described by Lamichhane et al. (2018c). Viable *Lactococcus lactis* cells were enumerated on M17 (Difco Laboratories; Detroit, MI) medium, supplemented with 0.5% (w/v) lactose, after aerobic incubation at 25 °C for 3 d (Ruggirello et al., 2018). Total numbers of *Lactobacillus helveticus* cells were enumerated on de Man, Rogosa, and Sharpe agar (BD, Oxford, UK) at pH 5.4 after anaerobic incubation for 3 d at 42 °C (Lamichhane et al., 2018c). Nonstarter lactic acid bacteria (NSLAB) cells were enumerated on *Lactobacillus* selection agar (BD), with an overlay, after aerobic incubation for 5 d at 30 °C (Lamichhane et al., 2018c).

2.4. *Proteolysis*

2.4.1. *pH 4.6-soluble nitrogen (% of total nitrogen)*

The levels of nitrogen soluble (expressed as % of total nitrogen) at pH 4.6 was measured after 1, 20, 48, and 90 d as described by Fenelon and Guinee (2000).

2.4.2. *Urea-polyacrylamide gel electrophoresis*

Urea-polyacrylamide gel electrophoresis (PAGE) of the cheeses at 1, 20, 48 and 90 d was performed, in duplicate, on a Protean II xi vertical slab gel unit (Biorad Laboratories Ltd., Watford, Herts, UK), as described by Sheehan and Guinee (2004a). Briefly, grated cheese samples (equivalent to 4 mg protein) were dissolved in 1 mL sample buffer, incubated at 55 °C for 10 min and each sample was loaded at a level of 12 µL per well. Sodium caseinate powder (Kerry Ingredients, Listowel) was used as an intact casein control. The samples ran initially through the stacking gel at 280 V and then through the separating gel at 300 V. The resulting gels were stained and scanned as described by McCarthy, Wilkinson, and Guinee (2017). Densitometry analysis was performed on the scanned images using image analysis software i.e., ImageJ (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). Eight major bands corresponding to caseins or its breakdown products were used for calculation: 1, β-casein(f106–209) (γ2); 2, β-casein(f29–209) (γ1); 3, β-casein(f108–209) (γ3); 4, β-casein; 5, β-casein(f1–192); 6, α_{S1}-casein; 7, α_{S1}-casein(f102–199); 8, α_{S1}-casein(f24–199). The area of each protein band was expressed as a percentage of total band area of these eight major bands. Levels of intact α_{S1}-casein and β-casein over ripening were expressed as a percentage of level at 1 d.

2.5. *Determination of total and insoluble calcium content*

The total calcium content of milk and cheese samples (after 20 d) was determined using atomic absorption spectroscopy (IDF, 2007). The cheese insoluble calcium contents,

expressed as percentage of total calcium, were determined after 1, 20, 48, and 90 d of ripening using an acid-base titration method as described by Hassan et al. (2004).

2.6. Fracture properties

Eight to 10 cylindrical samples (height 15 mm and diameter 12 mm) of each cheese were removed, using a borer and a wire cutter, at 20, 48 and 90 d of ripening. The cheese samples were wrapped in tin foil; half of the cylindrical cheese samples were stored at 4 °C and the remainder was stored at 23 °C for at least 4 hours. Cheese samples (at 4 °C or 23 °C) were compressed at a rate of 60 mm/min until fracture. True stress (σ; Equation 1) and Hencky strain ($\varepsilon_{H;}$ Equation 2) were calculated, assuming a constant volume deformation (Rehn et al., 2011):

$$\sigma = \frac{FH_t}{A_0 H_0} \qquad (1)$$

$$\varepsilon_H = \left| \ln \frac{H_t}{H_0} \right| \qquad (2)$$

where F is a load applied, H_t is the sample height at time t, and A_0 and H_0 are the initial cross-sectional area and height of sample, respectively. Fracture stress (σ_f) and fracture strain (ε_f) values of cheese samples were determined from the inflection point of the stress-strain curve (Rehn et al., 2011).

2.7. Visualization of cheese microstructure

Cheese microstructure was observed using cryogenic-scanning electron microscopy (cryo-SEM). This was conducted using an SEM system (SEM-Zeiss Supra 40VP field emission, Carl Zeiss AG, Darmstadt, Germany) with a cryogenic transfer system attached (Gatan Alto 2500, Gatan UK). Fresh cheese samples (after 90 d of ripening) were taken from the middle of each experimental cheese wheel and rapidly immersed into a liquid nitrogen slush (-200

°C) in a cryo-preparation chamber. The samples were transferred under vacuum into the high vacuum cryo-preparation chamber at -185 °C, etched at -95 °C over a period of 15 min, sputter-coated at -125 °C and finally transferred onto the SEM cold stage at -125 °C. Cryo-SEM images were acquired at -125 °C.

The microstructure of cheese samples was also visualised using confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems, Baden-Württemberg, Germany). Rectangular cheese samples (5 mm × 5 mm × 2 mm) were removed from cheeses using a sharp scalpel. Solutions of the protein specific dye Fast Green (Sigma Aldrich) and fat specific dye Nile Red (Sigma Aldrich) were prepared at a concentration of 0.01% (w/v) in 1,2-propanediol (Sigma Aldrich) and deionized water respectively, which were then mixed at a ratio of 3:1. The prepared dye mixture (40 μL) was applied to the surface of cheese samples; a cover slip was gently placed on top and the sample was held at 4 °C for 10 min prior to imaging. The protein and fat phases of the cheese samples were visualised by exciting the Fast Green dye (using a He–Ne laser; excitation wavelength of 633 nm and emission wavelength range of 650-700 nm) and Nile Red dye (using an Argon laser; excitation wavelength of 488 nm and emission wavelength range of 500-580 nm) respectively as described by Abhyankar, Mulvihill, and Auty (2014). All images were acquired using an oil immersion objective with a numerical aperture of 1.4 and a magnification of 63× (Leica Microsystems, Baden-Württemberg, Germany).

2.8. Statistical analysis

One way ANOVA, using SPSS software version 24 (IBM Corp., Armonk, NY), was performed to determine the effect of treatment on cheese composition. A split-plot design was used to determine the effect of treatment, ripening time, and their interactions on pH, counts of *Lactococcus lactis* and *Lactobacillus helveticus*, levels of pH 4.6-SN (% TN), insoluble calcium (% of total calcium) and fracture properties (stress and strain at fracture) of

cheese. Analysis for the split-plot design was carried out using the PROC MIXED procedure of SAS software version 9.3 (SAS Institute Inc., 2011). Tukey's multiple comparison tests was used for paired comparison of treatment means at a 5% level of significance. Pearson correlation analysis was performed between fracture parameters, pH 4.6-SN (% TN), insoluble calcium (% of total calcium), intact β-casein level and intact α_{S1}-casein level using SPSS software version 24 (IBM Corp., Armonk, NY).

3. Results and discussion

3.1. Milk and cheese composition

The average fat, protein, and lactose contents of the standardized and pasteurized cheese-milk used for the 3 replicate cheese-making trials were 3.21, 3.52, and 4.87 % (w/w), respectively. The composition of the experimental cheeses at 20 d of ripening is shown in Table 2. The cheeses had a composition similar to those of Maasdam-type cheese reported by Lamichhane et al. (2018a). The treatments applied had no significant effect on the mean levels of moisture, moisture in non-fat substance, protein, fat, fat-in-dry matter, salt, salt-in-moisture and pH (at 1 d of ripening) of the experimental cheeses.

3.2. pH

The pH of all experimental cheeses increased significantly ($P < 0.001$; Table 3) during ripening from 5.18-5.23 at 1 d to 5.35-5.40 at 90 d (Fig. 1a). The pH trend during ripening is consistent with that typical of washed-curd cheese types, such as Maasdam (Lamichhane et al., 2018a). No significant effect of treatment was observed for the mean value of pH during ripening.

3.3. Growth and viability of Lactococcus lactis, Lactobacillus helveticus and NSLAB

A significant effect of ripening time and treatment was observed for the counts of *Lactococcus lactis* (Table 3). The counts of *Lactococcus lactis* decreased in all cheeses

during ripening from $10^{9.4}$-$10^{9.7}$ cfu/g at 1 d to $10^{7.4}$-10^{9} cfu/g at 90 d, indicating cell death and potentially lysis of some *Lactococcus lactis* during ripening. Moreover, the count of *Lactococcus lactis* was significantly higher ($P < 0.05$) in noWR cheeses than other cheeses, suggesting that the death and possibly lysis of *Lactococcus lactis* was accelerated by the warm room ripening.

No significant effect of treatment and ripening time was observed for counts of *Lactobacillus helveticus* until 20 d of ripening, at which time the average count was 10^{5}-$10^{6.5}$ cfu/g. After warm-room ripening (48 d), the typical colonies of *Lactobacillus helveticus* were not observed, suggesting that either the cells were in a stressed condition which may be viable but not culturable, or may have lysed due to changes in the cheese-ripening environment, such as microbial composition, depletion of energy sources (e.g., low residual lactose), production of metabolites (Steele, Broadbent, & Kok, 2013) or inward diffusion of salt (Hickey, Fallico, Wilkinson, & Sheehan, 2018a).

NSLAB counts were variable between trials, although one trial did show that the average counts of NSLAB increased during ripening from $10^{4.3}$-10^{5} cfu/g at 20 d (before warm room ripening) to $10^{6.7}$-$10^{7.7}$ cfu/g at 48 d (after warm-room ripening). Moreover, the average count of NSLAB was ~1 log lower in noWR cheeses than for the other cheeses at 48 d of ripening.

3.4. Proteolysis

3.4.1. Nitrogen soluble at pH 4.6 (% of total nitrogen)

A significant ($P < 0.001$, Table 3) interaction was observed between the effect of treatment and ripening time for levels of nitrogen soluble at pH 4.6 [% of total nitrogen; pH 4.6-SN (% TN)] in all experimental cheeses. The mean levels of pH 4.6-SN (% TN) increased with increasing ripening time in all experimental cheeses (Fig. 1b). However, the extent of the increase in pH 4.6-SN (% TN) level during ripening was higher in control cheeses than for

other experimental cheese variants, which increased from 6.95 at 20 d to 19.27 at 90 d. The level of pH 4.6-SN (% TN) in control cheeses is in close agreement with that previously reported for semi-hard (Huc, Challois, Monziols, Michon, & Mariette, 2014) and Maasdam (Lamichhane et al., 2018a) cheeses.

Although propionic acid bacteria were not inoculated into the cheese milks of the current study, the levels and trend of pH 4.6-SN (% TN) during ripening of cheeses were found to be similar to semi-hard cheeses with propionic acid bacteria, suggesting that propionic acid bacteria have a minor role in the proteolysis of washed-curd brine-salted semi-hard cheese (Gagnaire, Thierry, & Léonil, 2001). Moreover, the autolysis of propionic acid bacteria and the release of proteases from their cell have been shown to be limited in cheese (Valence, Richoux, Thierry, Palva, & Lortal, 1998).

As expected, the mean level of pH 4.6-SN (% TN) in PepA cheeses was approximately two-fold lower that of control cheeses at 90 d; O'Mahony et al. (2005) has previously reported a similar trend for Cheddar cheese. The low level of proteolysis in the PepA cheeses is due to inhibition of residual chymosin by pepstatin A (which was added to the curd-whey mixture at a level of 10 μmol/L). The level of pH 4.6-SN (% TN) in PepA cheese was found similar to that reported for Emmental cheese at 90 d of ripening (O'Sullivan, McSweeney, Cotter, Giblin, & Sheehan, 2016); in Emmental, residual coagulant is largely or wholly inactivated by use of a high cook temperature during cheese manufacture.

The mean levels of pH 4.6-SN (% TN) in noWR and CC cheeses were 12.73 and 13.49, respectively, after 90 d of ripening, which were significantly lower than in the control cheeses. A higher average level of proteolysis in control cheeses compared to the noWR cheeses is attributed to an increase in the rate of proteolysis due to elevated ripening temperature (Sheehan et al., 2004b; Soodam, Ong, Powell, Kentish, & Gras, 2017). The lower levels of pH 4.6-SN (% TN) in CC cheese compared to control cheeses is attributed to

the lower general proteolytic activity of FPCC compared to FPBC (Kappeler et al., 2006; Bansal et al., 2009).

3.4.2. Urea-polyacrylamide gel electrophoresis

During ripening, α_{S1}- and β-caseins were hydrolyzed progressively to an extent dependent on the treatment applied and ripening temperature, while breakdown products accumulated simultaneously (Fig. 2 and Supplementary Fig. 1). Extensive hydrolysis of α_{S1}-casein was observed for control cheeses during ripening (i.e., more than 90 % of levels at 1 d), with the rate of hydrolysis being most rapid during warm room ripening stages, whereas the hydrolysis of α_{S1}-casein was ~30% and ~45% less in noWR and CC cheeses at 90 d, respectively, compared to control cheeses (Fig. 2b).

Less hydrolysis of α_{S1}-casein in noWR cheeses compared to control cheeses was attributed to the influence of temperature on the residual coagulant activity (Sheehan et al., 2004b). Less extensive breakdown of α_{S1}-casein in CC cheeses compared to control cheese is attributed to the lower proteolytic activity of FPCC compared to FPBC (Bansal et al., 2009; McCarthy et al., 2017).

Limited breakdown of α_{S1}-casein, i.e., ~5%, was observed in PepA cheeses in agreement with the previous studies (Shakeel-Ur-Rehman, Feeney, McSweeney, & Fox, 1998; O'Mahony et al., 2005), suggesting that the addition of chymosin inhibitor, i.e., pepstatin A, to the curd/whey mixture during cheese manufacture was an effective means for greatly reducing the chymosin-mediated hydrolysis of α_{S1}-casein within the semi-hard cheese during ripening.

Hydrolysis of β-casein was observed in all cheeses during ripening (Fig. 2c), most likely due to plasmin activity (Kelly et al., 2006). The extent of hydrolysis of β-casein was similar for control, CC and pepA cheeses (i.e., ~35 % of levels at 1 d), suggesting that neither the substitution of FPBC with FPCC nor addition of chymosin inhibitor to the curd/whey mixture

influenced the hydrolysis of β-casein in agreement with the previous studies (O'Mahony et al., 2005; Bansal et al., 2009). However, the extent of breakdown was relatively lower in noWR cheeses (i.e., less than 20% of levels at 1 d) than other cheeses, suggesting that warm room ripening accelerates the degradation of β-casein. Overall, these results suggest that the various hydrolysis patterns of casein can be achieved by using different coagulant types, modulating ripening temperature or inhibiting residual chymosin activity, although inhibition of the latter using pepstatin A is obviously not commercially viable.

3.5. Insoluble calcium contents of cheeses

The mean level of insoluble calcium (percentage of total calcium) decreased significantly ($P < 0.001$, Table 3) during ripening (Fig. 3), especially at the early stage of ripening, from ~75% at 1 d to ~66% at 20 d. After 20 d of ripening, the rate of decrease in the level of insoluble calcium was slower than at the early stages of ripening, which is in agreement with the previous studies in different cheese types (O'Mahony et al., 2005; Lee, Johnson, Govindasamy-Lucey, Jaeggi, & Lucey, 2010).

The effect of warm-room ripening on solubilization of colloidal calcium in brine-salted cheese varieties has not previously been studied. Therefore, the rate of calcium solubilization was compared between cheeses subjected to warm room ripening (control cheeses) and without warm room ripening (noWR cheeses). Interestingly, the mean insoluble calcium content of noWR cheeses was ~3% higher than that of the control cheese after 48 d of (after warm room ripening); however, the difference observed was not statistically significant, suggesting that, at best, the warm room ripening had only a minor effect on the solubilization of calcium. Hydrolysis of β-casein is known to release phosphopeptides (Gagnaire, Mollé, Herrouin, & Léonil, 2001), which could contribute to decreases in the level of casein-bound calcium. As expected, substitution of FPBC with FPCC as a coagulant or addition of

pepstatin A to the curd/whey mixture during cheese manufacture had no significant effect on insoluble calcium content.

3.6. Fracture properties

The fracture properties of experimental cheeses were studied at two different temperatures, i.e., 4 °C or 23 °C (Fig. 4). The stress at fracture (σ_f) and strain at fracture (ε_f) were significantly influenced by treatment and ripening time (Table 3).

Fracture stress (σ_f), the force required to cause fracture of cheese, represents the strength or rigidity of the cheese matrix. The σ_f measured at 4 °C or 23 °C decreased significantly (Fig. 4a-b; Table 3) in all cheeses over maturation. However, the σ_f was significantly higher ($P < 0.05$) in PepA, noWR and CC cheeses compared to control cheeses. A lower σ_f in the control cheeses compared to other experimental cheese types was attributed to higher levels of protein breakdown in the control compared to PepA, noWR and CC cheeses (Fig. 1b). A significant negative correlation (Table 4) between pH 4.6-SN (% TN) and σ_f was observed for the experimental cheeses, which is in agreement with previous studies on Cheddar cheese (McCarthy, Wilkinson, Kelly, & Guinee, 2016). Moreover, the σ_f value was significantly positively (Table 4) correlated with intact α_{S1}-casein. Intact β-casein level was also significantly positively correlated with the value of σ_f; however, the correlation coefficient (r) value was lower for intact β-casein (Table 4) as compared to intact α_{S1}-casein. This suggests that the intact α_{S1}-casein is the principle load-bearing protein within the semi-hard cheese matrix. No significant correlation was found between the σ_f and insoluble calcium content (Table 4), indicating that the extent of solubilization of calcium after 20 d of ripening had no pronounced influence on the strength of the cheese matrix.

Fracture strain (ε_f) represents the shortness or brittleness of cheese texture; cheeses with a lower fracture strain value are susceptible to fracture at small deformation (Grappin et al.,

1993; Sharma, Munro, Dessev, Wiles, & Foegeding, 2018). The ε_f measured at 4 °C or 23 °C decreased significantly for control, CC and PepA cheeses, especially during warm room ripening, from 1.0-1.2 at 20 d to 0.75-0.8 at 48 d (Fig. 4c-d).

Although α_{S1}-casein was hydrolyzed to varying degrees among the control, CC and PepA cheeses after 48 d of ripening (ranging from ~5% in PepA to ~90% in control cheeses; Fig. 2), no significant difference in ε_f was observed among these cheeses. In the current study, hydrolysis of α_{S1}-casein mainly occurred at Phe_{23}-Phe_{24} during ripening, yielding peptides α_{S1}-casein (f1-23) and α_{S1}-casein (f24-199). The former peptide may be hydrolyzed rapidly by proteinases of the starter micro-organisms (Shakeel-Ur-Rehman et al., 1998), whereas the latter peptide accumulated during ripening (Fig. 2a). Therefore, the results from this study suggest that the primary breakdown of α_{S1}-casein into the large peptide fragment, i.e., α_{S1}-casein (f24-199) had no pronounced effect on the ε_f in semi-hard cheese during ripening. Since the peptide fraction α_{S1}-casein (f24-199) is so large, it is likely that this fraction may remain attached to the protein network rather than becoming part of the serum phase (Luyten, 1988; Lucey et al., 2003). Further breakdown of α_{S1}-casein (f24-199) (secondary breakdown) into small peptides may decrease the ε_f of cheese (Luyten, 1988). In the current study, no noticeable breakdown of α_{S1}-casein (f24-199) was observed during 90 d of ripening (Fig. 2a); therefore, the role of secondary breakdown of α_{S1}-casein (f24-199) on shortness of cheese could not be elucidated. Similar to the current study, Luyten (1988) also didn't observe a clear link between the primary breakdown of α_{S1}-casein and ε_f in Gouda cheese. A significant decrease in ε_f in control, CC and PepA cheeses during warm-room ripening may be due to other age-related changes within the cheese matrix rather than primary breakdown of α_{S1}-casein.

Interestingly, the ε_f for the noWR cheeses remained almost the same or decreased slightly over the ripening period (Fig. 4c-d). Moreover, the ε_f for noWR cheeses was significantly

higher ($P < 0.05$) at 48 and 90 d as compared to control, PepA and CC cheeses (which were subjected to warm room ripening stage). Similarly, Luyten (1988) also observed considerably lower ε_f in Gouda cheeses ripened at higher temperature (i.e., 18 °C) than ripened at lower temperature (i.e., 8 °C) during ripening. Furthermore, similar to the current study, ε_f of the Gouda cheeses ripened at 8 °C decreased slightly from 1.3 at 14 d to 1.2 at 42 d of ripening, whereas ε_f of the Gouda cheese ripened at 18 °C decreased considerably from 1.3 to 0.8 over the same ripening period. Although the exact reasons for such an influence of ripening temperature on fracture behaviour of cheese are unknown, it may be assumed that temperature-induced changes within the cheese matrix, such as rate of solubilization of colloidal calcium, specific hydrolysis patterns of casein and the resultant peptide profiles, could be possible reasons.

In the current study, insoluble calcium (expressed as a percentage of total calcium) and intact β-casein were significantly positively correlated with ε_f (Table 4). Furthermore, levels of intact β-casein (Fig. 2c) and insoluble calcium (Fig. 3) were on average ~15% and ~3% higher, respectively, in noWR cheeses than in the other cheeses after 48 d of ripening. This suggests that the breakdown of intact β-casein, solubilization of colloidal calcium during ripening, or both may contribute to a shorter texture (i.e., lower ε_f) observed in control, CC and PepA than noWR cheeses. Therefore, the results from this study suggest that the influence of varying degrees of hydrolysis of β-casein or level of colloidal calcium on shortness of cheese texture merits further research.

It is now well established that the calcium associated with casein is an important structural component, which enhances the cross-linking of caseins within the cheese matrix (Lucey et al., 2003; O'Mahony et al., 2005; Lamichhane et al., 2018b). Thus, it is reasonable to assume that the solubilization of colloidal calcium during ripening within the cheese matrix is one of the possible reasons for shorter texture of cheese. Moreover, studies have suggested that the

caseins have different hydrophilic and hydrophobic blocks. For example, α_{S1}-casein has a hydrophilic region between strong hydrophobic regions, whereas the β-casein has a hydrophilic and a hydrophobic region at N and C termini, respectively (Lucey et al., 2003). Therefore, it is likely that the specific hydrolysis of caseins during ripening may alter their molecular interactions within cheese matrix which in turn may influence the texture, rheological and fracture behaviour of cheese. For example, Bogenrief and Olson (1995) observed a degree of melt of Cheddar cheese which was more closely related to the extent of β-CN hydrolysis than the hydrolysis of α_{S1}-CN.

Overall, the fracture behaviour of cheese can be modulated by specific hydrolysis of casein, modulation of colloidal calcium associated with casein, or both. Such knowledge is particularly important for designing cheese with desired texture profiles or for designing cheese texture suitable for withstanding increased gas pressures during ripening in some eye-type cheeses, which may help to reduce the incidence of undesirable splits and cracks (Daly et al., 2010). Studies have reported that the occurrence of cracks within the cheese matrix is higher for cheeses with lower ε_f (short or brittle texture) (Grappin et al., 1993; Rehn et al., 2011). However, it should be noted that unsuitable cheese texture is one possible contributing factor amongst other factors for the development of undesirable splits or cracks, such as; rate and extent of gas production and its behavior (e.g., solubility and diffusivity) within the cheese matrix; late gas production; and the presence of micro-defects within the cheese matrix (Daly et al., 2010).

The σ_f of cheeses measured at 4 °C (Fig. 4a) was considerably higher as compared to same cheeses measured at 23 °C (Fig. 4b) at all stages of ripening, which is attributed to the temperature-induced changes on the components of cheese and their interactions (Lamichhane et al., 2018b). At low temperature (~4 °C), more than half of the milk fat present within the cheese matrix is in a crystallized form, and acts as a reinforcing filler,

contributing to the elastic texture of cheese (Lopez, Briard-Bion, Camier, & Gassi, 2006; Lamichhane et al., 2018b). However, the test temperature (4 °C or 23 °C) had no pronounced effect on the ε_f of cheeses at all stages of ripening.

3.7. Microstructure

The microstructure of cheese (at 90 d of ripening) observed by cryo-SEM is shown in Fig. 5. The microstructure of the control cheese is clearly different from that of the other experimental cheese types; the microstructure observed for the control cheese was more open than that of the other experimental cheeses. The open structure may be attributed to significantly higher levels of proteolysis in the control cheeses compared to the other cheese types. For other experimental cheeses, the microstructure looks visually similar. During proteolysis, the intact caseins, which are responsible for network formation, breakdown into small and medium size peptides and free amino acids and these peptides and amino acids are released into the serum fraction of the cheese (Sousa, Ardö, & McSweeney, 2001). Soodam, Ong, Powell, Kentish, and Gras (2015) also observed a less open structure of cheese with low levels of primary proteolysis than in cheeses with high levels.

The microstructure of the cheeses (at 90 d ripening) was also visualized using CLSM (Supplementary Fig. 2). In agreement with the previous studies (Lopez, Camier, & Gassi, 2007), non-globular, coalesced and aggregated fat globules were observed within the cheese matrix, which is attributed to the aggregation, coalescence, and disruption of the fat globules due to the various cheese manufacture steps, such as cooking and pressing (Lopez et al., 2007). The microstructures of all experimental cheeses were visually similar.

4. Conclusions

The roles of primary proteolysis and calcium solubilization on the fracture properties of washed-curd brine-salted semi-hard cheese were investigated. Addition of a chymosin

inhibitor i.e., pepstatin A, to the curd/whey mixture during cheese manufacture, substitution of FPBC with FPCC or modulating ripening temperature altered the hydrolysis patterns of the caseins during ripening. Moreover, solubilization of colloidal calcium was also observed in all cheeses during ripening.

The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher stress at fracture) in cheeses with lower levels of proteolysis or higher levels of intact caseins, primarily α_{S1}-casein. However, contrary to expectation, shortness or brittleness (as indicated by lower strain at fracture) of cheese texture was negatively associated particularly with the level of intact β-casein and also with insoluble calcium content.

The results from this study suggest that modulation of hydrolysis of α_{S1}-casein is an effective means for maintaining the strength of the cheese matrix during ripening. This could be achieved by inhibition of residual chymosin activity, substitution of FPBC with FPCC or modulating ripening temperature. However, shortness or brittleness of cheese texture could potentially be altered by maintaining higher levels of intact β-casein or insoluble calcium content or both within the cheese matrix. Shortness or brittleness of cheese has previously been associated with undesirable slits or cracks. Therefore, the role of intact β-casein or insoluble calcium content on fracture behaviour, especially fracture strain, merits further research.

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Figure legends

Fig. 1. Age-related changes in the (a) pH and (b) level of nitrogen soluble at pH 4.6, expressed as percentage of total nitrogen, pH 4.6-SN (% TN). Data are the mean of data from three replicate trials; Error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fig. 2. (a) Urea-polyacrylamide gel electrophoretograms of semi-hard cheeses after 1, 20, 48 or 90 d. Sodium caseinate (lane NaCn) was included as an intact casein control. Protein bands were identified according to McCarthy et al. (2017): 1, β-casein(f106–209) (γ2); 2, β-casein(f29–209) (γ1); 3, β-casein(f108–209) (γ3); 4, β-casein; 5, β-casein(f1–192); 6, α_{S1}-casein; 7, α_{S1}-casein(f102–199); 8, α_{S1}-casein(f24–199). Level of (b) intact α_{S1}-casein and (c) intact β-casein as a percentage of the level at 1 d. Error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fig. 3. Changes in the percentage insoluble Ca (expressed as a percentage of total cheese Ca) as a function of ripening time in semi-hard cheeses. Data are the mean of data from three replicate trials and error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fig. 4. Changes in (a-b) fracture stress (σ_f, n = 2) and (c-d) fracture strain (ε_f, n = 3), measured at 4 °C (closed symbols) and 23 °C (open symbols), in semi-hard cheese during

ripening. Error bars represent standard error of mean. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fig. 5. Selected cryo-SEM micrographs of (a, e) Control, (b, f) noWR, (c, g) CC, and (d, h) PepA cheeses after 90 d of ripening. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A). P = protein matrix, F = fat globules, short arrows = spherical imprints in the protein matrix left by fat globules that were removed during sample preparation, and long arrows = remnant fat from globules partially removed during sample preparation.

Microstructure and fracture properties of semi-hard cheese: Differentiating the effects of primary proteolysis and calcium solubilization

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Abstract

The individual roles of hydrolysis of α_{S1}- and β-caseins, and calcium solubilization on the fracture properties of semi-hard cheeses, such as Maasdam and other eye-type cheeses, remain unclear. In this study, the hydrolysis patterns of casein were selectively altered by adding a chymosin inhibitor to the curd/whey mixture during cheese manufacture, by substituting fermentation-produced bovine chymosin (FPBC) with fermentation-produced camel chymosin (FPCC), or by modulating ripening temperature. Moreover, the level of insoluble calcium during ripening was quantified in all cheeses. Addition of a chymosin inhibitor, substitution of FPBC with FPCC, or ripening of cheeses at a consistent low temperature (8 °C) decreased the hydrolysis of α_{S1}-casein by ~95%, ~45%, or ~30%, respectively, after 90 d of ripening, whereas ~35% of β-casein was hydrolysed in that time for all cheeses, except for those ripened at a lower temperature (~17%). The proportion of insoluble calcium as a percentage of total calcium decreased significantly from ~75% to ~60% between 1 and 90 d. The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher fracture stress) in cheeses with lower levels of proteolysis or higher levels of intact caseins, primarily α_{S1}-casein. However, contrary to the expectation that shortness of cheese texture is associated with α_{S1}-casein hydrolysis, fracture strain was significantly positively correlated with the level of intact β-casein and insoluble calcium content, indicating that the cheeses with low levels of intact β-casein or insoluble calcium content were more likely to be shorter in texture (i.e., lower fracture strain). Overall, this study suggests that the fracture properties of cheese can be modified by selective hydrolysis of caseins, altering the level of insoluble calcium or both. Such approaches could be applied to design cheese with specific properties.

Keywords: Cheese; Proteolysis; Insoluble calcium; Fracture properties; Microstructure; Split or crack defect

1. Introduction

Knowledge of fracture properties of cheese is important for understanding breakdown properties of cheese during mastication, in designing cheese texture suitable for size reduction operations (e.g., slicing, dicing or grating), and in understanding the reasons for formation of undesirable texture defects within the cheese matrix, such as slits and cracks (Luyten, 1988).

Development of undesirable slits and cracks within the cheese matrix is an international problem in the manufacture of Swiss, Dutch and related eye-type cheeses, leading to downgrading of the product, resulting in lost revenue to manufacturers (Grappin, Lefier, Dasen, & Pochet, 1993; White, Broadbent, Oberg, & McMahon, 2003; Guggisberg et al., 2015). To date, the exact reasons for development of such defect are not known. However, excessive production of gas, an unsuitable cheese texture or both have been considered as root causes for occurrence of this defect (Daly, McSweeney, & Sheehan, 2010; Rehn et al., 2011). If the cheese texture is short or brittle (i.e., fracturing of cheese matrix at a relatively small deformation), the cheese matrix is no longer able to withstand increased gas pressure during eye-formation or storage, leading to formation of cracks and splits. Although the exact reasons for a cheese to become short or brittle during ripening are not yet fully understood, proteolysis, partial solubilization of colloidal calcium phosphate associated with *para*-casein matrix of the curd during ripening, or both, have been considered as possible reasons (Lucey, Johnson, & Horne, 2003; Daly et al., 2010). However, the role of primary proteolysis and level of insoluble calcium on fracture behaviour of brine-salted semi-hard cheese has not yet been fully elucidated.

From a structural perspective, α_{S1}-casein and β-casein are the two important caseins within the cheese matrix, and these undergo varying degree of hydrolysis during ripening in

different cheese varieties through the action of residual coagulant and plasmin, respectively (Sheehan, O'Sullivan, & Guinee, 2004b; Kelly, O'Flaherty, & Fox, 2006; Lamichhane, Kelly, & Sheehan, 2018b). Studies have suggested that the caseins have different hydrophilic and hydrophobic blocks. For example, α_{S1}-casein has a hydrophilic region between strong hydrophobic regions, whereas the β-casein has a hydrophilic and a hydrophobic region at N- and C-terminal, respectively (Lucey et al., 2003). Thus, these caseins are held together by various molecular forces within the cheese matrix. Moreover, calcium associated with casein enhances the cross-linking of casein within the cheese matrix. Therefore, it is reasonable to assume that both hydrolysis patterns of casein and solubilization of colloidal calcium during ripening alter the casein interactions, which may in turn influence the textural, rheological and fracture behaviour of cheese. A better understanding of the individual contribution of such factors may allow the development of specific strategies to design cheese with specific properties.

Unlike high maximum scald temperatures (~55 °C) in Emmental cheese manufacture, cheese curds are cooked only to ~40 °C during manufacture of most semi-hard cheeses, such as Maasdam and Jarlsberg (Fröhlich-Wyder et al., 2017), which is not sufficient to inactivate or reduce the residual chymosin activity, resulting in extensive breakdown of α_{S1}-casein during ripening (McGoldrick & Fox, 1999). The role of chymosin-mediated proteolysis on texture properties of Cheddar cheese has previously been studied by inhibition of the residual chymosin by the addition of a chymosin inhibitor to the curd-whey mixture (O'Mahony, Lucey, & McSweeney, 2005). However, little is known about the role of chymosin-mediated proteolysis on the fracture behavior of semi-hard Swiss, Dutch and related eye-type cheeses. Some semi-hard eye-type cheeses are ripened in a warm room (~23 °C) for 4-6 weeks for the development of eyes. However, the effect of such elevated ripening temperature on solubilization of calcium and hydrolysis of casein is also not fully understood.

The aim of this study was to decouple and explore the individual role of primary proteolysis (both of α_{S1}- and β-casein) and insoluble calcium on the fracture properties of washed-curd brine-salted semi-hard cheese.

2. Materials and methods

2.1. Milk supply and cheese manufacture

Raw milk was obtained from the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Ireland. Raw milk was first separated into skim milk and cream using bench top centrifugal separator. Using skim milk and cream, cheese milks were standardized to a protein to fat ratio of 1.10:1.00, with an average protein and fat content of 3.52 % (w/w) and 3.21 % (w/w), respectively. The standardized cheese-milks were then pasteurized at 72 °C for 15 sec (MicroThermics, USA) and stored at 4 °C overnight prior to cheese manufacture.

Washed-curd brine-salted semi-hard cheeses were manufactured in triplicate trials over a 3 month period. Standardized and pasteurized cheese milks were placed into jacketed cheese vats (Pierre Guerin Technologies, Niort, France) with each vat containing 11 kg cheese milk, for each replication. Each vat contained automated variable speed cutting and stirring equipment. All cheese milks were inoculated at 32 °C with frozen direct vat inoculation cultures: consisting of (1) R-604 (180 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*; and (2) LH-B02 (9 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactobacillus helveticus*. Propionic acid bacteria were not inoculated into the cheese milks to avoid subsequent eye-formation during ripening of cheese which would not permit measurement of texture parameters.

All cheese milks were pre-acidified to 6.55 using 4% (w/v) lactic acid (Sigma-Aldrich) prior to rennet addition. After 40 min of pre-ripening, the coagulant, fermentation-produced bovine chymosin (FPBC; CHY-MAX Plus, ~200 international milk clotting units (IMCU)/mL; Chr. Hansen Ltd., Cork, Ireland) was added at a level of 2 mL/11 kg cheese milk in 3 out of 4 vats, whereas fermentation-produced camel chymosin (FPCC; CHY-MAX M, ~200 IMCU/mL; Chr. Hansen Ltd., Cork, Ireland), was added at a level of 1.5 mL/11 kg cheese milk in the fourth vat. Coagulants were diluted ~1:10 with deionized water prior addition. The addition rates of both FPBC and FPCC to milk were predetermined through a series of rheological experiments where the levels of the coagulants were adjusted to achieve coagula of similar gel strength (35 Pa) after a set period of ~45 min.

All gels were cut at a constant firmness (G′) value of 35 Pa (as measured using a small-amplitude oscillatory rheometer, AR 2000ex, TA Instruments) and the resultant curd/whey mixture was allowed to heal for 5 min before being stirred continuously for another 10 min. Stirring was then stopped and a portion of whey (0.35 kg/kg cheese milk) was removed. Just after whey removal, in one vat out of four vats, Pepstatin A (synthetic; Enzo life science, Exeter, UK) was added to the curd/whey mixture at a rate of 10.0 μmol/kg cheese milk and evenly distributed by continuous stirring during cooking. Pepstatin A is an inhibitor of aspartic proteases, including chymosin, pepsin, cathepsin D, and renin (Marciniszyn, Hartsuck, & Tang, 1976). After whey removal, reverse osmosis water at ~50 °C (0.25 kg/kg cheese milk) was added to each cheese vat to cook the curd to 37 °C at a rate of 0.2 °C/min with continuous stirring.

Whey was drained when the curd pH reached 6.35, and the curds were collected into moulds and pressed vertically under increasing pressure from 40 to 75 kPa for ~4.5 hours. When the pH of the cheese curds reached ~5.50, the cheese wheels (~600 g each) were transferred to a saturated brine solution (23%, w/w, NaCl, 0.56%, w/w, $CaCl_2$, and pH 5.2) for 7.5 h at 8 °C.

After brining, cheese wheels were vacuum-packed (Falcon 52, Original Henkelman vacuum system, the Netherlands), and transferred to the ripening room. Cheese wheels were ripened at 8 °C for 20 d (pre-ripening), at 23 °C for 28 d (warm-room ripening) or 8 °C for 28 d (without warm room ripening), and finally stored at 4 °C for 42 d. A summary of the experimental plan is shown in Table 1.

2.2. Milk and cheese composition

The composition of raw and pasteurized (72 °C for 15 s) cheese milks were analyzed as described by Lamichhane, Kelly, and Sheehan (2018a). Grated cheese samples were analyzed at 20 d of ripening in duplicate for moisture, fat, protein and salt as described by Hickey et al. (2018b). Cheese pH was measured at 1, 20, 48 and 90 d as described by Sheehan, Fenelon, Wilkinson, and McSweeney (2007).

2.3. Enumeration of starter and nonstarter lactic acid bacteria

Samples were removed from cheese wheels using a cheese trier at 1, 20, 48 and 90 d of ripening. Cheese samples were prepared as described by Lamichhane et al. (2018c). Viable *Lactococcus lactis* cells were enumerated on M17 (Difco Laboratories; Detroit, MI) medium, supplemented with 0.5% (w/v) lactose, after aerobic incubation at 25 °C for 3 d (Ruggirello et al., 2018). Total numbers of *Lactobacillus helveticus* cells were enumerated on de Man, Rogosa, and Sharpe agar (BD, Oxford, UK) at pH 5.4 after anaerobic incubation for 3 d at 42 °C (Lamichhane et al., 2018c). Nonstarter lactic acid bacteria (NSLAB) cells were enumerated on *Lactobacillus* selection agar (BD), with an overlay, after aerobic incubation for 5 d at 30 °C (Lamichhane et al., 2018c).

2.4. *Proteolysis*

2.4.1. *pH 4.6-soluble nitrogen (% of total nitrogen)*

The levels of nitrogen soluble (expressed as % of total nitrogen) at pH 4.6 was measured after 1, 20, 48, and 90 d as described by Fenelon and Guinee (2000).

2.4.2. *Urea-polyacrylamide gel electrophoresis*

Urea-polyacrylamide gel electrophoresis (PAGE) of the cheeses at 1, 20, 48 and 90 d was performed, in duplicate, on a Protean II xi vertical slab gel unit (Biorad Laboratories Ltd., Watford, Herts, UK), as described by Sheehan and Guinee (2004a). Briefly, grated cheese samples (equivalent to 4 mg protein) were dissolved in 1 mL sample buffer, incubated at 55 °C for 10 min and each sample was loaded at a level of 12 µL per well. Sodium caseinate powder (Kerry Ingredients, Listowel) was used as an intact casein control. The samples ran initially through the stacking gel at 280 V and then through the separating gel at 300 V. The resulting gels were stained and scanned as described by McCarthy, Wilkinson, and Guinee (2017). Densitometry analysis was performed on the scanned images using image analysis software i.e., ImageJ (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). Eight major bands corresponding to caseins or its breakdown products were used for calculation: 1, β-casein(f106–209) (γ2); 2, β-casein(f29–209) (γ1); 3, β-casein(f108–209) (γ3); 4, β-casein; 5, β-casein(f1–192); 6, α_{S1}-casein; 7, α_{S1}-casein(f102–199); 8, α_{S1}-casein(f24–199). The area of each protein band was expressed as a percentage of total band area of these eight major bands. Levels of intact α_{S1}-casein and β-casein over ripening were expressed as a percentage of level at 1 d.

2.5. *Determination of total and insoluble calcium content*

The total calcium content of milk and cheese samples (after 20 d) was determined using atomic absorption spectroscopy (IDF, 2007). The cheese insoluble calcium contents,

expressed as percentage of total calcium, were determined after 1, 20, 48, and 90 d of ripening using an acid-base titration method as described by Hassan et al. (2004).

2.6. Fracture properties

Eight to 10 cylindrical samples (height 15 mm and diameter 12 mm) of each cheese were removed, using a borer and a wire cutter, at 20, 48 and 90 d of ripening. The cheese samples were wrapped in tin foil; half of the cylindrical cheese samples were stored at 4 °C and the remainder was stored at 23 °C for at least 4 hours. Cheese samples (at 4 °C or 23 °C) were compressed at a rate of 60 mm/min until fracture. True stress (σ; Equation 1) and Hencky strain ($\varepsilon_{H;}$ Equation 2) were calculated, assuming a constant volume deformation (Rehn et al., 2011):

$$\sigma = \frac{FH_t}{A_0 H_0} \qquad (1)$$

$$\varepsilon_H = \left| \ln \frac{H_t}{H_0} \right| \qquad (2)$$

where F is a load applied, H_t is the sample height at time t, and A_0 and H_0 are the initial cross-sectional area and height of sample, respectively. Fracture stress (σ_f) and fracture strain (ε_f) values of cheese samples were determined from the inflection point of the stress-strain curve (Rehn et al., 2011).

2.7. Visualization of cheese microstructure

Cheese microstructure was observed using cryogenic-scanning electron microscopy (cryo-SEM). This was conducted using an SEM system (SEM-Zeiss Supra 40VP field emission, Carl Zeiss AG, Darmstadt, Germany) with a cryogenic transfer system attached (Gatan Alto 2500, Gatan UK). Fresh cheese samples (after 90 d of ripening) were taken from the middle of each experimental cheese wheel and rapidly immersed into a liquid nitrogen slush (-200

°C) in a cryo-preparation chamber. The samples were transferred under vacuum into the high vacuum cryo-preparation chamber at -185 °C, etched at -95 °C over a period of 15 min, sputter-coated at -125 °C and finally transferred onto the SEM cold stage at -125 °C. Cryo-SEM images were acquired at -125 °C.

The microstructure of cheese samples was also visualised using confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems, Baden-Württemberg, Germany). Rectangular cheese samples (5 mm × 5 mm × 2 mm) were removed from cheeses using a sharp scalpel. Solutions of the protein specific dye Fast Green (Sigma Aldrich) and fat specific dye Nile Red (Sigma Aldrich) were prepared at a concentration of 0.01% (w/v) in 1,2-propanediol (Sigma Aldrich) and deionized water respectively, which were then mixed at a ratio of 3:1. The prepared dye mixture (40 µL) was applied to the surface of cheese samples; a cover slip was gently placed on top and the sample was held at 4 °C for 10 min prior to imaging. The protein and fat phases of the cheese samples were visualised by exciting the Fast Green dye (using a He–Ne laser; excitation wavelength of 633 nm and emission wavelength range of 650-700 nm) and Nile Red dye (using an Argon laser; excitation wavelength of 488 nm and emission wavelength range of 500-580 nm) respectively as described by Abhyankar, Mulvihill, and Auty (2014). All images were acquired using an oil immersion objective with a numerical aperture of 1.4 and a magnification of 63× (Leica Microsystems, Baden-Württemberg, Germany).

2.8. Statistical analysis

One way ANOVA, using SPSS software version 24 (IBM Corp., Armonk, NY), was performed to determine the effect of treatment on cheese composition. A split-plot design was used to determine the effect of treatment, ripening time, and their interactions on pH, counts of *Lactococcus lactis* and *Lactobacillus helveticus*, levels of pH 4.6-SN (% TN), insoluble calcium (% of total calcium) and fracture properties (stress and strain at fracture) of

cheese. Analysis for the split-plot design was carried out using the PROC MIXED procedure of SAS software version 9.3 (SAS Institute Inc., 2011). Tukey's multiple comparison tests was used for paired comparison of treatment means at a 5% level of significance. Pearson correlation analysis was performed between fracture parameters, pH 4.6-SN (% TN), insoluble calcium (% of total calcium), intact β-casein level and intact α_{S1}-casein level using SPSS software version 24 (IBM Corp., Armonk, NY).

3. Results and discussion

3.1. Milk and cheese composition

The average fat, protein, and lactose contents of the standardized and pasteurized cheese-milk used for the 3 replicate cheese-making trials were 3.21, 3.52, and 4.87 % (w/w), respectively. The composition of the experimental cheeses at 20 d of ripening is shown in Table 2. The cheeses had a composition similar to those of Maasdam-type cheese reported by Lamichhane et al. (2018a). The treatments applied had no significant effect on the mean levels of moisture, moisture in non-fat substance, protein, fat, fat-in-dry matter, salt, salt-in-moisture and pH (at 1 d of ripening) of the experimental cheeses.

3.2. pH

The pH of all experimental cheeses increased significantly ($P < 0.001$; Table 3) during ripening from 5.18-5.23 at 1 d to 5.35-5.40 at 90 d (Fig. 1a). The pH trend during ripening is consistent with that typical of washed-curd cheese types, such as Maasdam (Lamichhane et al., 2018a). No significant effect of treatment was observed for the mean value of pH during ripening.

3.3. Growth and viability of Lactococcus lactis, Lactobacillus helveticus and NSLAB

A significant effect of ripening time and treatment was observed for the counts of *Lactococcus lactis* (Table 3). The counts of *Lactococcus lactis* decreased in all cheeses

during ripening from $10^{9.4}$-$10^{9.7}$ cfu/g at 1 d to $10^{7.4}$-10^{9} cfu/g at 90 d, indicating cell death and potentially lysis of some *Lactococcus lactis* during ripening. Moreover, the count of *Lactococcus lactis* was significantly higher ($P < 0.05$) in noWR cheeses than other cheeses, suggesting that the death and possibly lysis of *Lactococcus lactis* was accelerated by the warm room ripening.

No significant effect of treatment and ripening time was observed for counts of *Lactobacillus helveticus* until 20 d of ripening, at which time the average count was 10^{5}-$10^{6.5}$ cfu/g. After warm-room ripening (48 d), the typical colonies of *Lactobacillus helveticus* were not observed, suggesting that either the cells were in a stressed condition which may be viable but not culturable, or may have lysed due to changes in the cheese-ripening environment, such as microbial composition, depletion of energy sources (e.g., low residual lactose), production of metabolites (Steele, Broadbent, & Kok, 2013) or inward diffusion of salt (Hickey, Fallico, Wilkinson, & Sheehan, 2018a).

NSLAB counts were variable between trials, although one trial did show that the average counts of NSLAB increased during ripening from $10^{4.3}$-10^{5} cfu/g at 20 d (before warm room ripening) to $10^{6.7}$-$10^{7.7}$ cfu/g at 48 d (after warm-room ripening). Moreover, the average count of NSLAB was ~1 log lower in noWR cheeses than for the other cheeses at 48 d of ripening.

3.4. Proteolysis

3.4.1. Nitrogen soluble at pH 4.6 (% of total nitrogen)

A significant ($P < 0.001$, Table 3) interaction was observed between the effect of treatment and ripening time for levels of nitrogen soluble at pH 4.6 [% of total nitrogen; pH 4.6-SN (% TN)] in all experimental cheeses. The mean levels of pH 4.6-SN (% TN) increased with increasing ripening time in all experimental cheeses (Fig. 1b). However, the extent of the increase in pH 4.6-SN (% TN) level during ripening was higher in control cheeses than for

other experimental cheese variants, which increased from 6.95 at 20 d to 19.27 at 90 d. The level of pH 4.6-SN (% TN) in control cheeses is in close agreement with that previously reported for semi-hard (Huc, Challois, Monziols, Michon, & Mariette, 2014) and Maasdam (Lamichhane et al., 2018a) cheeses.

Although propionic acid bacteria were not inoculated into the cheese milks of the current study, the levels and trend of pH 4.6-SN (% TN) during ripening of cheeses were found to be similar to semi-hard cheeses with propionic acid bacteria, suggesting that propionic acid bacteria have a minor role in the proteolysis of washed-curd brine-salted semi-hard cheese (Gagnaire, Thierry, & Léonil, 2001). Moreover, the autolysis of propionic acid bacteria and the release of proteases from their cell have been shown to be limited in cheese (Valence, Richoux, Thierry, Palva, & Lortal, 1998).

As expected, the mean level of pH 4.6-SN (% TN) in PepA cheeses was approximately two-fold lower that of control cheeses at 90 d; O'Mahony et al. (2005) has previously reported a similar trend for Cheddar cheese. The low level of proteolysis in the PepA cheeses is due to inhibition of residual chymosin by pepstatin A (which was added to the curd-whey mixture at a level of 10 μmol/L). The level of pH 4.6-SN (% TN) in PepA cheese was found similar to that reported for Emmental cheese at 90 d of ripening (O'Sullivan, McSweeney, Cotter, Giblin, & Sheehan, 2016); in Emmental, residual coagulant is largely or wholly inactivated by use of a high cook temperature during cheese manufacture.

The mean levels of pH 4.6-SN (% TN) in noWR and CC cheeses were 12.73 and 13.49, respectively, after 90 d of ripening, which were significantly lower than in the control cheeses. A higher average level of proteolysis in control cheeses compared to the noWR cheeses is attributed to an increase in the rate of proteolysis due to elevated ripening temperature (Sheehan et al., 2004b; Soodam, Ong, Powell, Kentish, & Gras, 2017). The lower levels of pH 4.6-SN (% TN) in CC cheese compared to control cheeses is attributed to

the lower general proteolytic activity of FPCC compared to FPBC (Kappeler et al., 2006; Bansal et al., 2009).

3.4.2. Urea-polyacrylamide gel electrophoresis

During ripening, α_{S1}- and β-caseins were hydrolyzed progressively to an extent dependent on the treatment applied and ripening temperature, while breakdown products accumulated simultaneously (Fig. 2 and Supplementary Fig. 1). Extensive hydrolysis of α_{S1}-casein was observed for control cheeses during ripening (i.e., more than 90 % of levels at 1 d), with the rate of hydrolysis being most rapid during warm room ripening stages, whereas the hydrolysis of α_{S1}-casein was ~30% and ~45% less in noWR and CC cheeses at 90 d, respectively, compared to control cheeses (Fig. 2b).

Less hydrolysis of α_{S1}-casein in noWR cheeses compared to control cheeses was attributed to the influence of temperature on the residual coagulant activity (Sheehan et al., 2004b). Less extensive breakdown of α_{S1}-casein in CC cheeses compared to control cheese is attributed to the lower proteolytic activity of FPCC compared to FPBC (Bansal et al., 2009; McCarthy et al., 2017).

Limited breakdown of α_{S1}-casein, i.e., ~5%, was observed in PepA cheeses in agreement with the previous studies (Shakeel-Ur-Rehman, Feeney, McSweeney, & Fox, 1998; O'Mahony et al., 2005), suggesting that the addition of chymosin inhibitor, i.e., pepstatin A, to the curd/whey mixture during cheese manufacture was an effective means for greatly reducing the chymosin-mediated hydrolysis of α_{S1}-casein within the semi-hard cheese during ripening.

Hydrolysis of β-casein was observed in all cheeses during ripening (Fig. 2c), most likely due to plasmin activity (Kelly et al., 2006). The extent of hydrolysis of β-casein was similar for control, CC and pepA cheeses (i.e., ~35 % of levels at 1 d), suggesting that neither the substitution of FPBC with FPCC nor addition of chymosin inhibitor to the curd/whey mixture

influenced the hydrolysis of β-casein in agreement with the previous studies (O'Mahony et al., 2005; Bansal et al., 2009). However, the extent of breakdown was relatively lower in noWR cheeses (i.e., less than 20% of levels at 1 d) than other cheeses, suggesting that warm room ripening accelerates the degradation of β-casein. Overall, these results suggest that the various hydrolysis patterns of casein can be achieved by using different coagulant types, modulating ripening temperature or inhibiting residual chymosin activity, although inhibition of the latter using pepstatin A is obviously not commercially viable.

3.5. Insoluble calcium contents of cheeses

The mean level of insoluble calcium (percentage of total calcium) decreased significantly ($P < 0.001$, Table 3) during ripening (Fig. 3), especially at the early stage of ripening, from ~75% at 1 d to ~66% at 20 d. After 20 d of ripening, the rate of decrease in the level of insoluble calcium was slower than at the early stages of ripening, which is in agreement with the previous studies in different cheese types (O'Mahony et al., 2005; Lee, Johnson, Govindasamy-Lucey, Jaeggi, & Lucey, 2010).

The effect of warm-room ripening on solubilization of colloidal calcium in brine-salted cheese varieties has not previously been studied. Therefore, the rate of calcium solubilization was compared between cheeses subjected to warm room ripening (control cheeses) and without warm room ripening (noWR cheeses). Interestingly, the mean insoluble calcium content of noWR cheeses was ~3% higher than that of the control cheese after 48 d of (after warm room ripening); however, the difference observed was not statistically significant, suggesting that, at best, the warm room ripening had only a minor effect on the solubilization of calcium. Hydrolysis of β-casein is known to release phosphopeptides (Gagnaire, Mollé, Herrouin, & Léonil, 2001), which could contribute to decreases in the level of casein-bound calcium. As expected, substitution of FPBC with FPCC as a coagulant or addition of

pepstatin A to the curd/whey mixture during cheese manufacture had no significant effect on insoluble calcium content.

3.6. Fracture properties

The fracture properties of experimental cheeses were studied at two different temperatures, i.e., 4 °C or 23 °C (Fig. 4). The stress at fracture (σ_f) and strain at fracture (ε_f) were significantly influenced by treatment and ripening time (Table 3).

Fracture stress (σ_f), the force required to cause fracture of cheese, represents the strength or rigidity of the cheese matrix. The σ_f measured at 4 °C or 23 °C decreased significantly (Fig. 4a-b; Table 3) in all cheeses over maturation. However, the σ_f was significantly higher ($P < 0.05$) in PepA, noWR and CC cheeses compared to control cheeses. A lower σ_f in the control cheeses compared to other experimental cheese types was attributed to higher levels of protein breakdown in the control compared to PepA, noWR and CC cheeses (Fig. 1b). A significant negative correlation (Table 4) between pH 4.6-SN (% TN) and σ_f was observed for the experimental cheeses, which is in agreement with previous studies on Cheddar cheese (McCarthy, Wilkinson, Kelly, & Guinee, 2016). Moreover, the σ_f value was significantly positively (Table 4) correlated with intact α_{S1}-casein. Intact β-casein level was also significantly positively correlated with the value of σ_f; however, the correlation coefficient (r) value was lower for intact β-casein (Table 4) as compared to intact α_{S1}-casein. This suggests that the intact α_{S1}-casein is the principle load-bearing protein within the semi-hard cheese matrix. No significant correlation was found between the σ_f and insoluble calcium content (Table 4), indicating that the extent of solubilization of calcium after 20 d of ripening had no pronounced influence on the strength of the cheese matrix.

Fracture strain (ε_f) represents the shortness or brittleness of cheese texture; cheeses with a lower fracture strain value are susceptible to fracture at small deformation (Grappin et al.,

1993; Sharma, Munro, Dessev, Wiles, & Foegeding, 2018). The ε_f measured at 4 °C or 23 °C decreased significantly for control, CC and PepA cheeses, especially during warm room ripening, from 1.0-1.2 at 20 d to 0.75-0.8 at 48 d (Fig. 4c-d).

Although α_{S1}-casein was hydrolyzed to varying degrees among the control, CC and PepA cheeses after 48 d of ripening (ranging from ~5% in PepA to ~90% in control cheeses; Fig. 2), no significant difference in ε_f was observed among these cheeses. In the current study, hydrolysis of α_{S1}-casein mainly occurred at Phe_{23}-Phe_{24} during ripening, yielding peptides α_{S1}-casein (f1-23) and α_{S1}-casein (f24-199). The former peptide may be hydrolyzed rapidly by proteinases of the starter micro-organisms (Shakeel-Ur-Rehman et al., 1998), whereas the latter peptide accumulated during ripening (Fig. 2a). Therefore, the results from this study suggest that the primary breakdown of α_{S1}-casein into the large peptide fragment, i.e., α_{S1}-casein (f24-199) had no pronounced effect on the ε_f in semi-hard cheese during ripening. Since the peptide fraction α_{S1}-casein (f24-199) is so large, it is likely that this fraction may remain attached to the protein network rather than becoming part of the serum phase (Luyten, 1988; Lucey et al., 2003). Further breakdown of α_{S1}-casein (f24-199) (secondary breakdown) into small peptides may decrease the ε_f of cheese (Luyten, 1988). In the current study, no noticeable breakdown of α_{S1}-casein (f24-199) was observed during 90 d of ripening (Fig. 2a); therefore, the role of secondary breakdown of α_{S1}-casein (f24-199) on shortness of cheese could not be elucidated. Similar to the current study, Luyten (1988) also didn't observe a clear link between the primary breakdown of α_{S1}-casein and ε_f in Gouda cheese. A significant decrease in ε_f in control, CC and PepA cheeses during warm-room ripening may be due to other age-related changes within the cheese matrix rather than primary breakdown of α_{S1}-casein.

Interestingly, the ε_f for the noWR cheeses remained almost the same or decreased slightly over the ripening period (Fig. 4c-d). Moreover, the ε_f for noWR cheeses was significantly

higher ($P < 0.05$) at 48 and 90 d as compared to control, PepA and CC cheeses (which were subjected to warm room ripening stage). Similarly, Luyten (1988) also observed considerably lower ε_f in Gouda cheeses ripened at higher temperature (i.e., 18 °C) than ripened at lower temperature (i.e., 8 °C) during ripening. Furthermore, similar to the current study, ε_f of the Gouda cheeses ripened at 8 °C decreased slightly from 1.3 at 14 d to 1.2 at 42 d of ripening, whereas ε_f of the Gouda cheese ripened at 18 °C decreased considerably from 1.3 to 0.8 over the same ripening period. Although the exact reasons for such an influence of ripening temperature on fracture behaviour of cheese are unknown, it may be assumed that temperature-induced changes within the cheese matrix, such as rate of solubilization of colloidal calcium, specific hydrolysis patterns of casein and the resultant peptide profiles, could be possible reasons.

In the current study, insoluble calcium (expressed as a percentage of total calcium) and intact β-casein were significantly positively correlated with ε_f (Table 4). Furthermore, levels of intact β-casein (Fig. 2c) and insoluble calcium (Fig. 3) were on average ~15% and ~3% higher, respectively, in noWR cheeses than in the other cheeses after 48 d of ripening. This suggests that the breakdown of intact β-casein, solubilization of colloidal calcium during ripening, or both may contribute to a shorter texture (i.e., lower ε_f) observed in control, CC and PepA than noWR cheeses. Therefore, the results from this study suggest that the influence of varying degrees of hydrolysis of β-casein or level of colloidal calcium on shortness of cheese texture merits further research.

It is now well established that the calcium associated with casein is an important structural component, which enhances the cross-linking of caseins within the cheese matrix (Lucey et al., 2003; O'Mahony et al., 2005; Lamichhane et al., 2018b). Thus, it is reasonable to assume that the solubilization of colloidal calcium during ripening within the cheese matrix is one of the possible reasons for shorter texture of cheese. Moreover, studies have suggested that the

caseins have different hydrophilic and hydrophobic blocks. For example, α_{S1}-casein has a hydrophilic region between strong hydrophobic regions, whereas the β-casein has a hydrophilic and a hydrophobic region at N and C termini, respectively (Lucey et al., 2003). Therefore, it is likely that the specific hydrolysis of caseins during ripening may alter their molecular interactions within cheese matrix which in turn may influence the texture, rheological and fracture behaviour of cheese. For example, Bogenrief and Olson (1995) observed a degree of melt of Cheddar cheese which was more closely related to the extent of β-CN hydrolysis than the hydrolysis of α_{S1}-CN.

Overall, the fracture behaviour of cheese can be modulated by specific hydrolysis of casein, modulation of colloidal calcium associated with casein, or both. Such knowledge is particularly important for designing cheese with desired texture profiles or for designing cheese texture suitable for withstanding increased gas pressures during ripening in some eye-type cheeses, which may help to reduce the incidence of undesirable splits and cracks (Daly et al., 2010). Studies have reported that the occurrence of cracks within the cheese matrix is higher for cheeses with lower ε_f (short or brittle texture) (Grappin et al., 1993; Rehn et al., 2011). However, it should be noted that unsuitable cheese texture is one possible contributing factor amongst other factors for the development of undesirable splits or cracks, such as; rate and extent of gas production and its behavior (e.g., solubility and diffusivity) within the cheese matrix; late gas production; and the presence of micro-defects within the cheese matrix (Daly et al., 2010).

The σ_f of cheeses measured at 4 °C (Fig. 4a) was considerably higher as compared to same cheeses measured at 23 °C (Fig. 4b) at all stages of ripening, which is attributed to the temperature-induced changes on the components of cheese and their interactions (Lamichhane et al., 2018b). At low temperature (~4 °C), more than half of the milk fat present within the cheese matrix is in a crystallized form, and acts as a reinforcing filler,

contributing to the elastic texture of cheese (Lopez, Briard-Bion, Camier, & Gassi, 2006; Lamichhane et al., 2018b). However, the test temperature (4 °C or 23 °C) had no pronounced effect on the ε_f of cheeses at all stages of ripening.

3.7. Microstructure

The microstructure of cheese (at 90 d of ripening) observed by cryo-SEM is shown in Fig. 5. The microstructure of the control cheese is clearly different from that of the other experimental cheese types; the microstructure observed for the control cheese was more open than that of the other experimental cheeses. The open structure may be attributed to significantly higher levels of proteolysis in the control cheeses compared to the other cheese types. For other experimental cheeses, the microstructure looks visually similar. During proteolysis, the intact caseins, which are responsible for network formation, breakdown into small and medium size peptides and free amino acids and these peptides and amino acids are released into the serum fraction of the cheese (Sousa, Ardö, & McSweeney, 2001). Soodam, Ong, Powell, Kentish, and Gras (2015) also observed a less open structure of cheese with low levels of primary proteolysis than in cheeses with high levels.

The microstructure of the cheeses (at 90 d ripening) was also visualized using CLSM (Supplementary Fig. 2). In agreement with the previous studies (Lopez, Camier, & Gassi, 2007), non-globular, coalesced and aggregated fat globules were observed within the cheese matrix, which is attributed to the aggregation, coalescence, and disruption of the fat globules due to the various cheese manufacture steps, such as cooking and pressing (Lopez et al., 2007). The microstructures of all experimental cheeses were visually similar.

4. Conclusions

The roles of primary proteolysis and calcium solubilization on the fracture properties of washed-curd brine-salted semi-hard cheese were investigated. Addition of a chymosin

inhibitor i.e., pepstatin A, to the curd/whey mixture during cheese manufacture, substitution of FPBC with FPCC or modulating ripening temperature altered the hydrolysis patterns of the caseins during ripening. Moreover, solubilization of colloidal calcium was also observed in all cheeses during ripening.

The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher stress at fracture) in cheeses with lower levels of proteolysis or higher levels of intact caseins, primarily α_{S1}-casein. However, contrary to expectation, shortness or brittleness (as indicated by lower strain at fracture) of cheese texture was negatively associated particularly with the level of intact β-casein and also with insoluble calcium content.

The results from this study suggest that modulation of hydrolysis of α_{S1}-casein is an effective means for maintaining the strength of the cheese matrix during ripening. This could be achieved by inhibition of residual chymosin activity, substitution of FPBC with FPCC or modulating ripening temperature. However, shortness or brittleness of cheese texture could potentially be altered by maintaining higher levels of intact β-casein or insoluble calcium content or both within the cheese matrix. Shortness or brittleness of cheese has previously been associated with undesirable slits or cracks. Therefore, the role of intact β-casein or insoluble calcium content on fracture behaviour, especially fracture strain, merits further research.

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Figure legends

Fig. 1. Age-related changes in the (a) pH and (b) level of nitrogen soluble at pH 4.6, expressed as percentage of total nitrogen, pH 4.6-SN (% TN). Data are the mean of data from three replicate trials. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fig. 2. (a) Urea-polyacrylamide gel electrophoretograms of semi-hard cheeses after 1, 20, 48 or 90 d. Sodium caseinate (lane NaCn) was included as an intact casein control. Protein bands were identified according to McCarthy et al. (2017): 1, β-casein(f106–209) (γ2); 2, β-casein(f29–209) (γ1); 3, β-casein(f108–209) (γ3); 4, β-casein; 5, β-casein(f1–192); 6, α_{S1}-casein; 7, α_{S1}-casein(f102–199); 8, α_{S1}-casein(f24–199). Level of (b) intact α_{S1}-casein and (c) intact β-casein as a percentage of the level at 1 d. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fig. 3. Changes in the percentage insoluble Ca (expressed as a percentage of total cheese Ca) as a function of ripening time in semi-hard cheeses. Data are the mean of data from three replicate trials and error bars show the standard error of mean from 3 replicate trials. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fig. 4. Changes in (a-b) fracture stress (σ_f, n = 2) and (c-d) fracture strain (ε_f, n = 3), measured at 4 °C (closed symbols) and 23 °C (open symbols), in semi-hard cheese during

ripening. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

Fig. 5. Selected cryo-SEM micrographs of (a, e) Control, (b, f) noWR, (c, g) CC, and (d, h) PepA cheeses after 90 d of ripening. Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A). P = protein matrix, F = fat globules, short arrows = spherical imprints in the protein matrix left by fat globules that were removed during sample preparation, and long arrows = remnant fat from globules partially removed during sample preparation.

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Figure

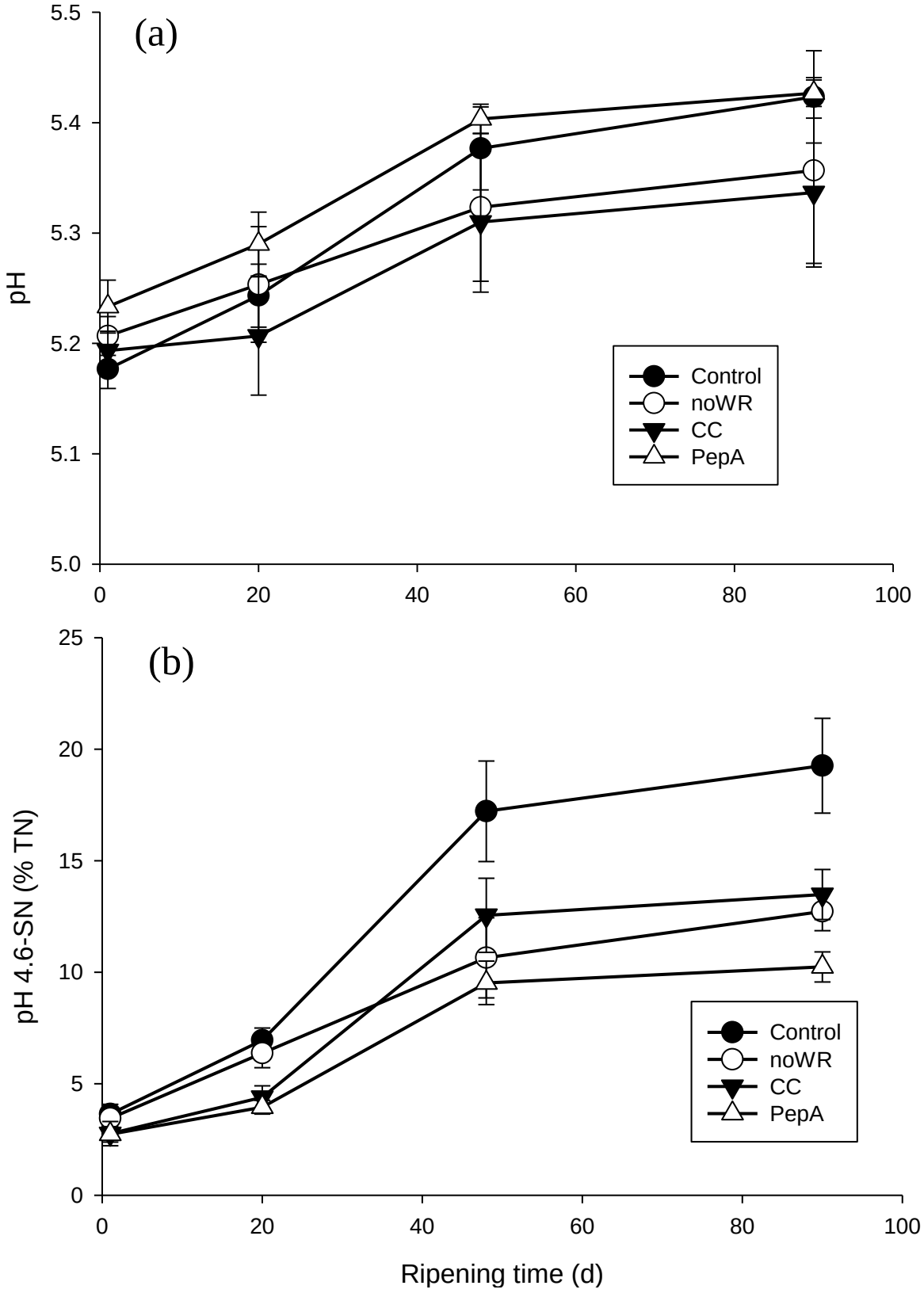

Figure 1.

Figure 2

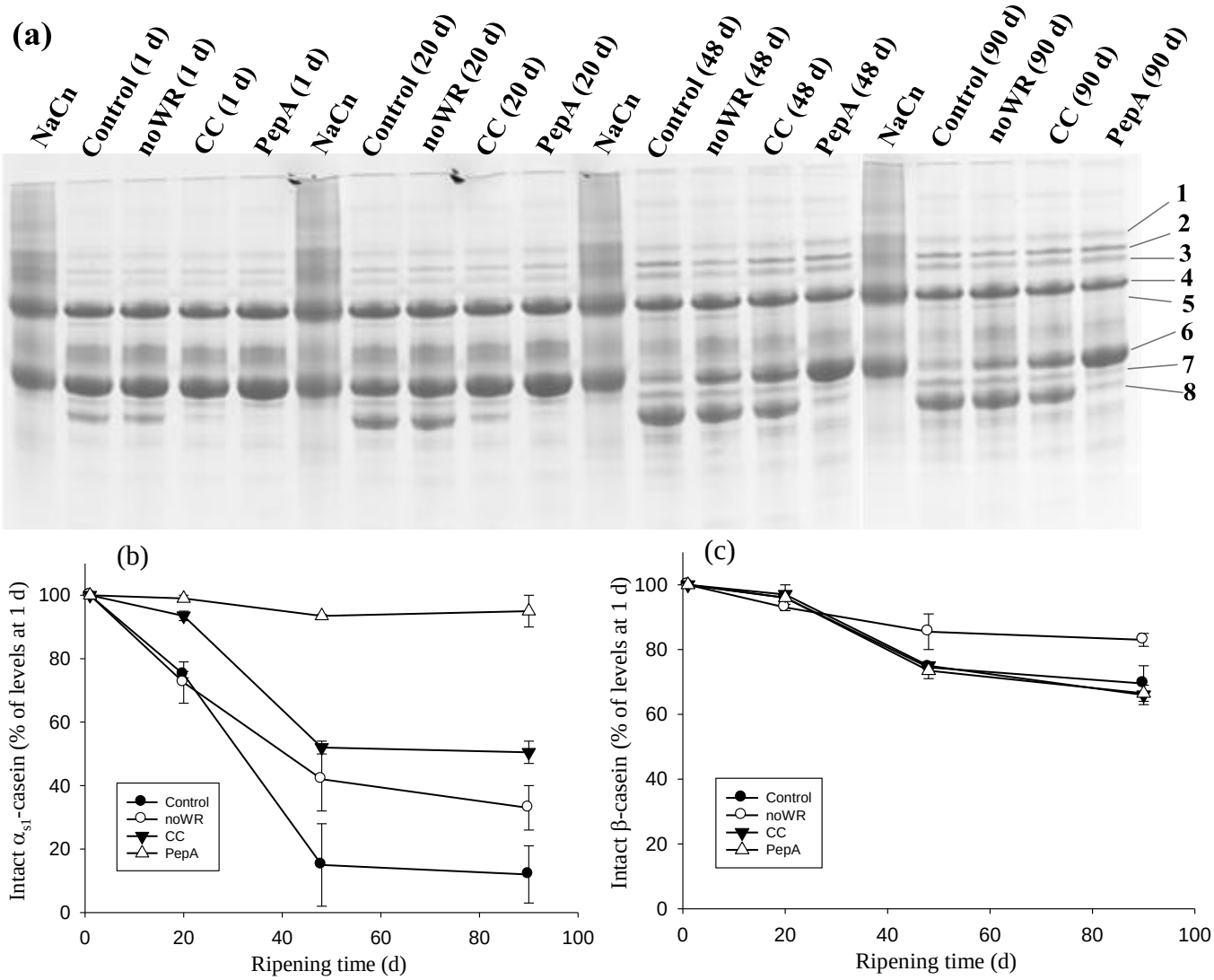

Figure 2.

Figure 3

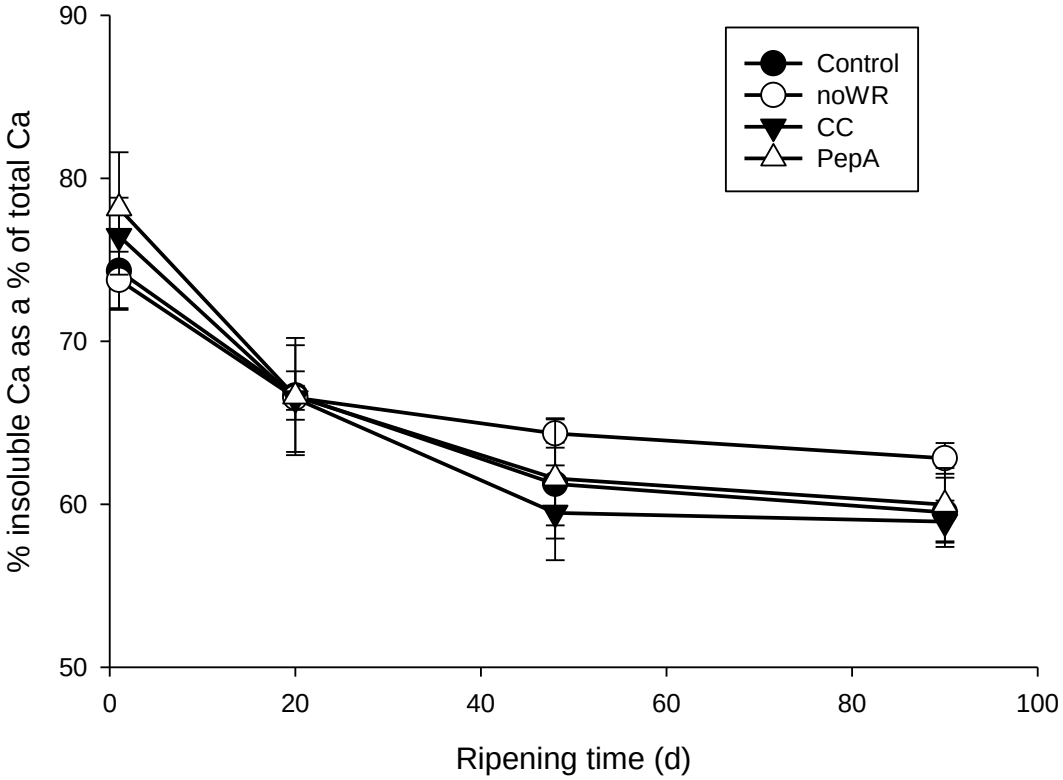

Figure 3.

Figure 4

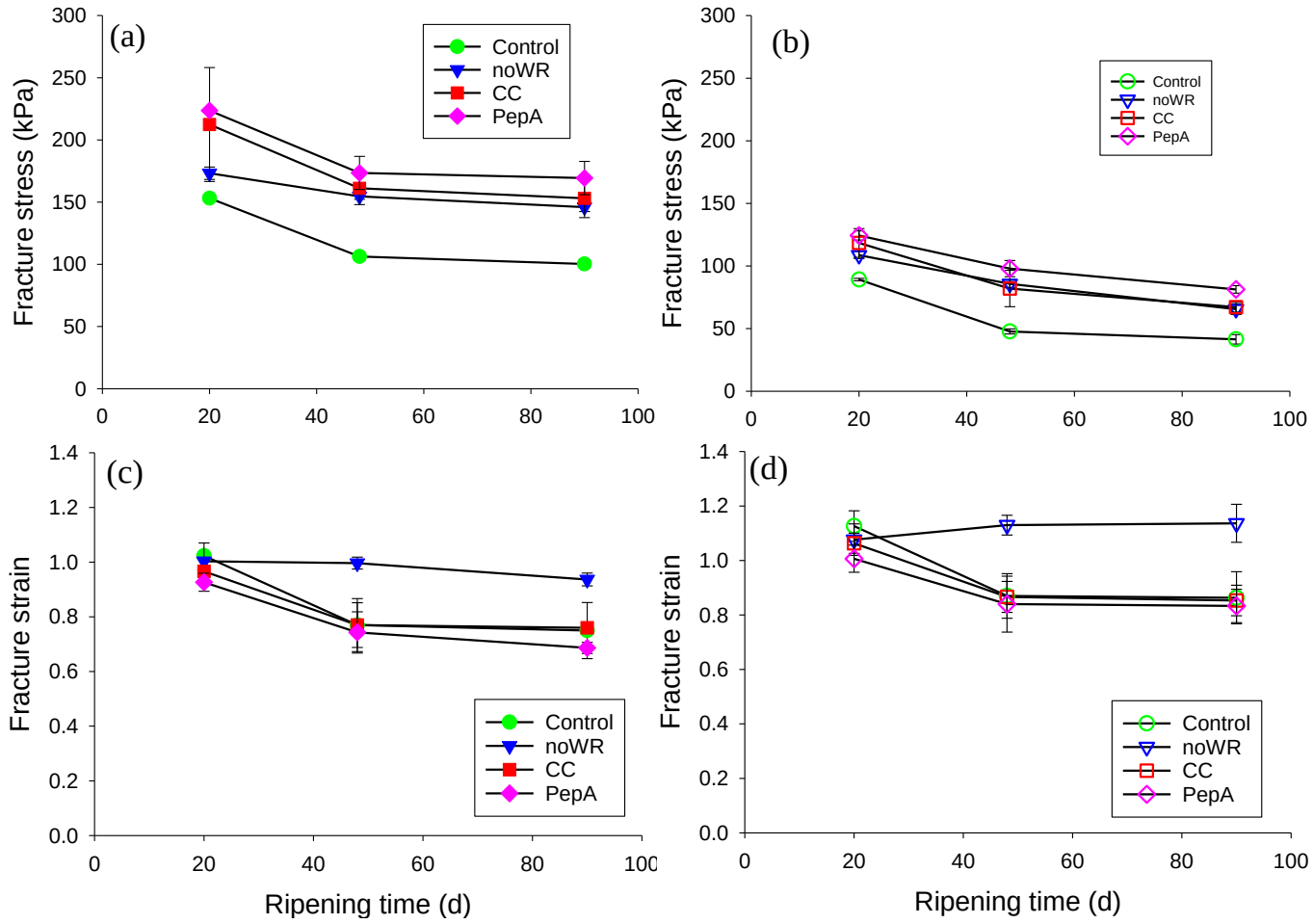

Figure 4.

Figure 5

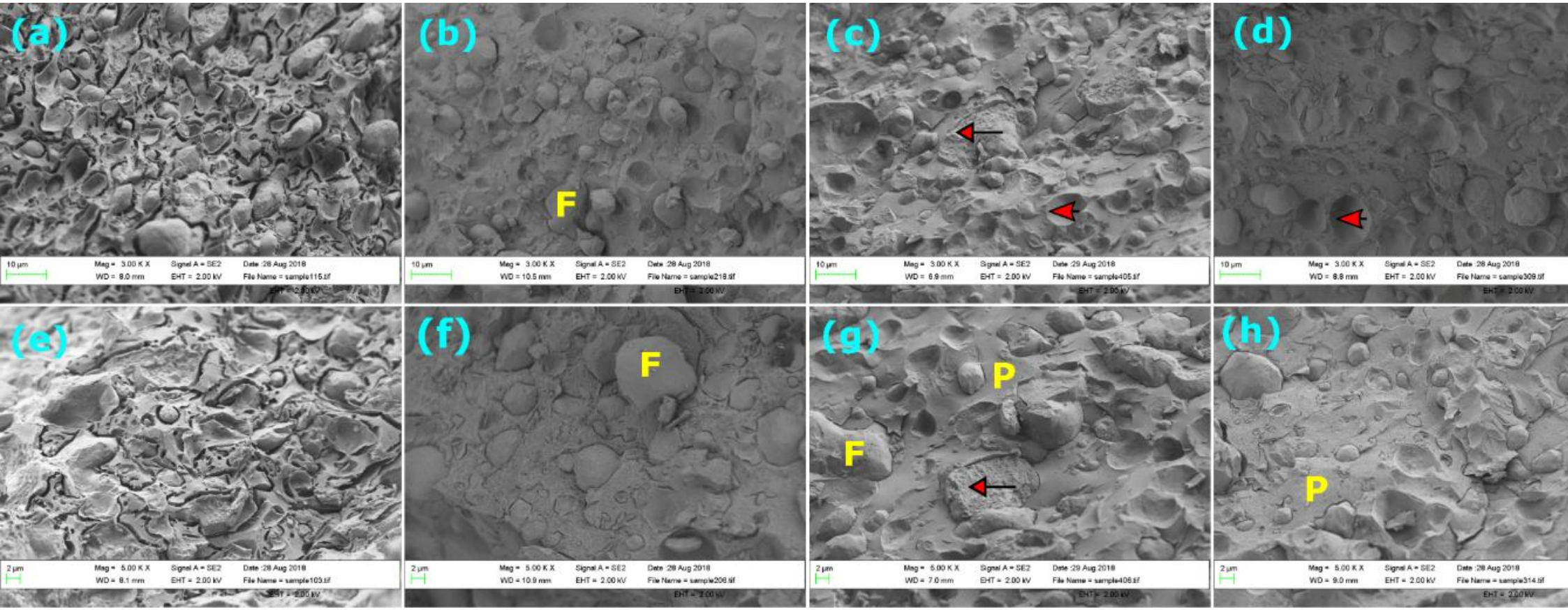

Fig. 5.

Table 1

Table 1. General overview of the treatments and ripening regimens used in the study[1]

Treatment	Cheese type[2]			
	Control	noWR	CC	PepA
Rennet type	FPBC	FPBC	**FPCC**	FPBC
Chymosin inhibitor	Not added	Not added	Not added	**Added**
Ripening regimen	8 °C for 20 d	8 °C for 20 d	8 °C for 20 d	8 °C for 20 d
	23 °C for 28 d	**8 °C for 28 d**	23 °C for 28 d	23 °C for 28 d
	4 °C for 39 d	4 °C for 39 d	4 °C for 39 d	4 °C for 39 d

[1]FPBC, fermentation-produced bovine chymosin; FPCC, fermentation-produced camel chymosin

[2]noWR, cheese without warm room ripening; CC, cheese made using fermentation- produced camel chymosin as a coagulant; PepA, cheese containing chymosin inhibitor, i.e., pepstatin A, which was added to the curd/whey mixture during cheese manufacture.

Table 2

Table 2. Compositional parameters at 20 d and pH at 1 d of ripening in semi-hard cheeses[1]

Compositional factors	Cheese types[2]				*P*-value
	Control	noWR	CC	PepA	
Moisture (%, w/w)	40.83 ± 1.61[a]	40.89 ± 2.47[a]	40.95 ± 3.36[a]	41.84 ± 2.51[a]	0.96
MNFS (%, w/w)	56.24 ± 1.54[a]	56.11 ± 2.20[a]	56.26 ± 3.11[a]	57.05 ± 2.28[a]	0.96
Protein (%, w/w)	25.32 ± 0.90[a]	25.57 ± 1.47[a]	25.44 ± 1.56[a]	25.39 ± 1.48[a]	0.98
Fat (%, w/w)	27.42 ± 1.06[a]	27.16 ± 1.77[a]	27.29 ± 1.98[a]	26.70 ± 1.65[a]	0.95
FDM (%, w/w)	46.33 ± 0.91[a]	45.92 ± 1.46[a]	46.18 ± 0.85[a]	45.88 ± 1.28[a]	0.96
Salt (%, w/w)	1.34 ± 0.12[a]	1.38 ± 0.14[a]	1.38 ± 0.09[a]	1.36 ± 0.08[a]	0.96
S/M (%, w/w)	3.28 ± 0.31[a]	3.39 ± 0.51[a]	3.38 ± 0.41[a]	3.25 ± 0.08[a]	0.95
Total calcium (mg/100 g cheese)	867 ± 30[a]	861 ± 30[a]	837 ± 34[a]	842 ± 27[a]	0.60
pH (1 d)	5.18 ± 0.03[a]	5.21 ± 0.03[a]	5.19 ± 0.03[a]	5.23 ± 0.03[a]	0.28

[1]MNFS, moisture in non-fat substance; FDM, fat in dry matter; S/M, salt-to-moisture ratio; Control, control cheeses; noWR, cheeses without warm room ripening; CC, cheeses made using fermentation-produced camel chymosin; PepA, cheeses containing chymosin inhibitor i.e., pepstatin A, which was added to the curd/whey mixture during cheese manufacture.

[2]Values within a row not sharing common superscripts differ ($P < 0.05$); data are the mean ± standard deviation of data from three replicate trials.

Table 3

Table 3. Summary of the effects of treatment, time and their interactions on properties of semi-hard cheeses[1]

Parameter	Treatment	Time	Interactive effect (treatment × time)
pH	NS	***	NS
Lactococcus lactis count	**	***	NS
Lactobacillus helveticus count	NS	NS	NS
pH 4.6-SN (% TN)	***	***	***
Insoluble Ca (% of total Ca)	NS	***	NS
Fracture stress (kPa, measured at 4 °C)	***	**	NS
Fracture stress (kPa, measured at 23 °C)	***	***	NS
Fracture strain (measured at 4 °C)	**	***	NS
Fracture strain (measured at 23 °C)	**	**	NS

[1]pH 4.6-SN (% TN), soluble nitrogen at pH 4.6 as percentage of total nitrogen.

**$P < 0.01$; ***$P < 0.001$; NS, $P > 0.05$

Table 4

Table. 4. Pearson correlation coefficients between fracture parameters, primary proteolysis, insoluble calcium, and intact β-casein and α_{S1}-casein[1]

	σ_f (measured at 4 °C)	ε_f (measured at 4 °C)	σ_f (measured at 23 °C)	ε_f (measured at 23 °C)
pH 4.6-SN (% TN)	-0.77**	-0.62**	-0.88**	-0.54**
Insoluble Ca (% of total Ca)	0.23^{NS}	0.66**	0.33^{NS}	0.53**
Intact β-casein	0.50*	0.75**	0.68**	0.60**
Intact α_{S1}-casein	0.79**	0.25^{NS}	0.83**	0.19^{NS}

[1]σ_f, stress at fracture; ε_f, strain at fracture; data were obtained from all experimental cheeses over a 90 d of ripening.

** $P < 0.01$; *$P < 0.05$; $^{NS}P > 0.05$

***Graphical Abstract**

Graphical abstract