

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.

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

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11 Abstract

12 The individual roles of hydrolysis of α_{S1} - and β -caseins, and calcium solubilization on the
13 fracture properties of semi-hard cheeses, such as Maasdam and other eye-type cheeses,
14 remain unclear. In this study, the hydrolysis patterns of casein were selectively altered by
15 adding a chymosin inhibitor to the curd/whey mixture during cheese manufacture, by
16 substituting fermentation-produced bovine chymosin (FPBC) with fermentation-produced
17 camel chymosin (FPCC), or by modulating ripening temperature. Moreover, the level of
18 insoluble calcium during ripening was quantified in all cheeses. Addition of a chymosin
19 inhibitor, substitution of FPBC with FPCC, or ripening of cheeses at a consistent low
20 temperature (8 °C) decreased the hydrolysis of α_{S1} -casein by ~95%, ~45%, or ~30%,
21 respectively, after 90 d of ripening, whereas ~35% of β -casein was hydrolysed in that time
22 for all cheeses, except for those ripened at a lower temperature (~17%). The proportion of
23 insoluble calcium as a percentage of total calcium decreased significantly from ~75% to
24 ~60% between 1 and 90 d. The rigidity or strength of the cheese matrix was found to be
25 higher (as indicated by higher fracture stress) in cheeses with lower levels of proteolysis or
26 higher levels of intact caseins, primarily α_{S1} -casein. However, contrary to the expectation that
27 shortness of cheese texture is associated with α_{S1} -casein hydrolysis, fracture strain was
28 significantly positively correlated with the level of intact β -casein and insoluble calcium
29 content, indicating that the cheeses with low levels of intact β -casein or insoluble calcium
30 content were more likely to be shorter in texture (i.e., lower fracture strain). Overall, this
31 study suggests that the fracture properties of cheese can be modified by selective hydrolysis
32 of caseins, altering the level of insoluble calcium or both. Such approaches could be applied
33 to design cheese with specific properties.

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

1 36 **1. Introduction**

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3 37 Knowledge of fracture properties of cheese is important for understanding breakdown
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6 38 properties of cheese during mastication, in designing cheese texture suitable for size
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8 39 reduction operations (e.g., slicing, dicing or grating), and in understanding the reasons for
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10 40 formation of undesirable texture defects within the cheese matrix, such as slits and cracks
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12 41 (Luyten, 1988).
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16 42 Development of undesirable slits and cracks within the cheese matrix is an international
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18 43 problem in the manufacture of Swiss, Dutch and related eye-type cheeses, leading to
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21 44 downgrading of the product, resulting in lost revenue to manufacturers (Grappin, Lefier,
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23 45 Dasen, & Pochet, 1993; White, Broadbent, Oberg, & McMahon, 2003; Guggisberg et al.,
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25 46 2015). To date, the exact reasons for development of such defect are not known. However,
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27 47 excessive production of gas, an unsuitable cheese texture or both have been considered as
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29 48 root causes for occurrence of this defect (Daly, McSweeney, & Sheehan, 2010; Rehn et al.,
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31 49 2011). If the cheese texture is short or brittle (i.e., fracturing of cheese matrix at a relatively
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33 50 small deformation), the cheese matrix is no longer able to withstand increased gas pressure
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35 51 during eye-formation or storage, leading to formation of cracks and splits. Although the exact
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37 52 reasons for a cheese to become short or brittle during ripening are not yet fully understood,
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39 53 proteolysis, partial solubilization of colloidal calcium phosphate associated with *para*-casein
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41 54 matrix of the curd during ripening, or both, have been considered as possible reasons (Lucey,
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43 55 Johnson, & Horne, 2003; Daly et al., 2010). However, the role of primary proteolysis and
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45 56 level of insoluble calcium on fracture behaviour of brine-salted semi-hard cheese has not yet
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47 57 been fully elucidated.
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51 58 From a structural perspective, α_{S1} -casein and β -casein are the two important caseins within
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53 59 the cheese matrix, and these undergo varying degree of hydrolysis during ripening in
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60 different cheese varieties through the action of residual coagulant and plasmin, respectively
61 (Sheehan, O'Sullivan, & Guinee, 2004b; Kelly, O'Flaherty, & Fox, 2006; Lamichhane,
62 Kelly, & Sheehan, 2018b). Studies have suggested that the caseins have different hydrophilic
63 and hydrophobic blocks. For example, α_{S1} -casein has a hydrophilic region between strong
64 hydrophobic regions, whereas the β -casein has a hydrophilic and a hydrophobic region at N-
65 and C-terminal, respectively (Lucey et al., 2003). Thus, these caseins are held together by
66 various molecular forces within the cheese matrix. Moreover, calcium associated with casein
67 enhances the cross-linking of casein within the cheese matrix. Therefore, it is reasonable to
68 assume that both hydrolysis patterns of casein and solubilization of colloidal calcium during
69 ripening alter the casein interactions, which may in turn influence the textural, rheological
70 and fracture behaviour of cheese. A better understanding of the individual contribution of
71 such factors may allow the development of specific strategies to design cheese with specific
72 properties.

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

1 85 The aim of this study was to decouple and explore the individual role of primary proteolysis
2 86 (both of α_{S1} - and β -casein) and insoluble calcium on the fracture properties of washed-curd
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4 87 brine-salted semi-hard cheese.
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8 88 **2. Materials and methods**

9 10 89 *2.1. Milk supply and cheese manufacture*

11 90 Raw milk was obtained from the Teagasc Animal and Grassland Research and Innovation
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13 91 Centre, Moorepark, Ireland. Raw milk was first separated into skim milk and cream using
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16 92 bench top centrifugal separator. Using skim milk and cream, cheese milks were standardized
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18 93 to a protein to fat ratio of 1.10:1.00, with an average protein and fat content of 3.52 % (w/w)
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20 94 and 3.21 % (w/w), respectively. The standardized cheese-milks were then pasteurized at 72
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22 95 °C for 15 sec (MicroThermics, USA) and stored at 4 °C overnight prior to cheese
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24 96 manufacture.
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31 97 Washed-curd brine-salted semi-hard cheeses were manufactured in triplicate trials over a 3
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33 98 month period. Standardized and pasteurized cheese milks were placed into jacketed cheese
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35 99 vats (Pierre Guerin Technologies, Niort, France) with each vat containing 11 kg cheese milk,
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38 100 for each replication. Each vat contained automated variable speed cutting and stirring
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40 101 equipment. All cheese milks were inoculated at 32 °C with frozen direct vat inoculation
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42 102 cultures: consisting of (1) R-604 (180 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland),
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44 103 containing *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*; and (2) LH-B02
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46 104 (9 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactobacillus helveticus*.
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49 105 Propionic acid bacteria were not inoculated into the cheese milks to avoid subsequent eye-
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51 106 formation during ripening of cheese which would not permit measurement of texture
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108 All cheese milks were pre-acidified to 6.55 using 4% (w/v) lactic acid (Sigma-Aldrich) prior
109 to rennet addition. After 40 min of pre-ripening, the coagulant, fermentation-produced bovine
110 chymosin (FPBC; CHY-MAX Plus, ~200 international milk clotting units (IMCU)/mL; Chr.
111 Hansen Ltd., Cork, Ireland) was added at a level of 2 mL/11 kg cheese milk in 3 out of 4 vats,
112 whereas fermentation-produced camel chymosin (FPCC; CHY-MAX M, ~200 IMCU/mL;
113 Chr. Hansen Ltd., Cork, Ireland), was added at a level of 1.5 mL/11 kg cheese milk in the
114 fourth vat. Coagulants were diluted ~1:10 with deionized water prior addition. The addition
115 rates of both FPBC and FPCC to milk were predetermined through a series of rheological
116 experiments where the levels of the coagulants were adjusted to achieve coagula of similar
117 gel strength (35 Pa) after a set period of ~45 min.

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

129 Whey was drained when the curd pH reached 6.35, and the curds were collected into moulds
130 and pressed vertically under increasing pressure from 40 to 75 kPa for ~4.5 hours. When the
131 pH of the cheese curds reached ~5.50, the cheese wheels (~600 g each) were transferred to a
132 saturated brine solution (23%, w/w, NaCl, 0.56%, w/w, CaCl₂, and pH 5.2) for 7.5 h at 8 °C.

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

138 2.2. *Milk and cheese composition*

139 The composition of raw and pasteurized (72 °C for 15 s) cheese milks were analyzed as
140 described by Lamichhane, Kelly, and Sheehan (2018a). Grated cheese samples were analyzed
141 at 20 d of ripening in duplicate for moisture, fat, protein and salt as described by Hickey et al.
142 (2018b). Cheese pH was measured at 1, 20, 48 and 90 d as described by Sheehan, Fenelon,
143 Wilkinson, and McSweeney (2007).

144 2.3. *Enumeration of starter and nonstarter lactic acid bacteria*

145 Samples were removed from cheese wheels using a cheese trier at 1, 20, 48 and 90 d of
146 ripening. Cheese samples were prepared as described by Lamichhane et al. (2018c). Viable
147 *Lactococcus lactis* cells were enumerated on M17 (Difco Laboratories; Detroit, MI) medium,
148 supplemented with 0.5% (w/v) lactose, after aerobic incubation at 25 °C for 3 d (Ruggirello
149 et al., 2018). Total numbers of *Lactobacillus helveticus* cells were enumerated on de Man,
150 Rogosa, and Sharpe agar (BD, Oxford, UK) at pH 5.4 after anaerobic incubation for 3 d at 42
151 °C (Lamichhane et al., 2018c). Nonstarter lactic acid bacteria (NSLAB) cells were
152 enumerated on *Lactobacillus* selection agar (BD), with an overlay, after aerobic incubation
153 for 5 d at 30 °C (Lamichhane et al., 2018c).

154 2.4. *Proteolysis*

155 2.4.1. *pH 4.6-soluble nitrogen (% of total nitrogen)*

156 The levels of nitrogen soluble (expressed as % of total nitrogen) at pH 4.6 was measured after
157 1, 20, 48, and 90 d as described by Fenelon and Guinee (2000).

158 2.4.2. *Urea-polyacrylamide gel electrophoresis*

159 Urea-polyacrylamide gel electrophoresis (PAGE) of the cheeses at 1, 20, 48 and 90 d was
160 performed, in duplicate, on a Protean II xi vertical slab gel unit (Biorad Laboratories Ltd.,
161 Watford, Herts, UK), as described by Sheehan and Guinee (2004a). Briefly, grated cheese
162 samples (equivalent to 4 mg protein) were dissolved in 1 mL sample buffer, incubated at 55
163 °C for 10 min and each sample was loaded at a level of 12 µL per well. Sodium caseinate
164 powder (Kerry Ingredients, Listowel) was used as an intact casein control. The samples ran
165 initially through the stacking gel at 280 V and then through the separating gel at 300 V. The
166 resulting gels were stained and scanned as described by McCarthy, Wilkinson, and Guinee
167 (2017). Densitometry analysis was performed on the scanned images using image analysis
168 software i.e., ImageJ (NIH, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>). Eight major
169 bands corresponding to caseins or its breakdown products were used for calculation: 1, β-
170 casein(f106–209) (γ2); 2, β-casein(f29–209) (γ1); 3, β-casein(f108–209) (γ3); 4, β-casein; 5,
171 β-casein(f1–192); 6, α_{S1}-casein; 7, α_{S1}-casein(f102–199); 8, α_{S1}-casein(f24–199). The area of
172 each protein band was expressed as a percentage of total band area of these eight major
173 bands. Levels of intact α_{S1}-casein and β-casein over ripening were expressed as a percentage
174 of level at 1 d.

175 2.5. *Determination of total and insoluble calcium content*

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

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

180 2.6. *Fracture properties*

181 Eight to 10 cylindrical samples (height 15 mm and diameter 12 mm) of each cheese were
182 removed, using a borer and a wire cutter, at 20, 48 and 90 d of ripening. The cheese samples
183 were wrapped in tin foil; half of the cylindrical cheese samples were stored at 4 °C and the
184 remainder was stored at 23 °C for at least 4 hours. Cheese samples (at 4 °C or 23 °C) were
185 compressed at a rate of 60 mm/min until fracture. True stress (σ ; Equation 1) and Hencky
186 strain (ε_H ; Equation 2) were calculated, assuming a constant volume deformation (Rehn et al.,
187 2011):

$$188 \quad \sigma = \frac{FH_t}{A_0H_0} \quad (1)$$

$$189 \quad \varepsilon_H = \left| \ln \frac{H_t}{H_0} \right| \quad (2)$$

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

194 2.7. *Visualization of cheese microstructure*

195 Cheese microstructure was observed using cryogenic-scanning electron microscopy (cryo-
196 SEM). This was conducted using an SEM system (SEM-Zeiss Supra 40VP field emission,
197 Carl Zeiss AG, Darmstadt, Germany) with a cryogenic transfer system attached (Gatan Alto
198 2500, Gatan UK). Fresh cheese samples (after 90 d of ripening) were taken from the middle
199 of each experimental cheese wheel and rapidly immersed into a liquid nitrogen slush (-200

200 °C) in a cryo-preparation chamber. The samples were transferred under vacuum into the high
201 vacuum cryo-preparation chamber at -185 °C, etched at -95 °C over a period of 15 min,
202 sputter-coated at -125 °C and finally transferred onto the SEM cold stage at -125 °C. Cryo-
203 SEM images were acquired at -125 °C.

204 The microstructure of cheese samples was also visualised using confocal laser scanning
205 microscopy (Leica TCS SP5, Leica Microsystems, Baden-Württemberg, Germany).
206 Rectangular cheese samples (5 mm × 5 mm × 2 mm) were removed from cheeses using a
207 sharp scalpel. Solutions of the protein specific dye Fast Green (Sigma Aldrich) and fat
208 specific dye Nile Red (Sigma Aldrich) were prepared at a concentration of 0.01% (w/v) in
209 1,2-propanediol (Sigma Aldrich) and deionized water respectively, which were then mixed at
210 a ratio of 3:1. The prepared dye mixture (40 µL) was applied to the surface of cheese
211 samples; a cover slip was gently placed on top and the sample was held at 4 °C for 10 min
212 prior to imaging. The protein and fat phases of the cheese samples were visualised by
213 exciting the Fast Green dye (using a He-Ne laser; excitation wavelength of 633 nm and
214 emission wavelength range of 650-700 nm) and Nile Red dye (using an Argon laser;
215 excitation wavelength of 488 nm and emission wavelength range of 500-580 nm) respectively
216 as described by Abhyankar, Mulvihill, and Auty (2014). All images were acquired using an
217 oil immersion objective with a numerical aperture of 1.4 and a magnification of 63× (Leica
218 Microsystems, Baden-Württemberg, Germany).

219 2.8. *Statistical analysis*

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

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

231 **3. Results and discussion**

232 *3.1. Milk and cheese composition*

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

240 *3.2. pH*

241 The pH of all experimental cheeses increased significantly ($P < 0.001$; Table 3) during
242 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
243 consistent with that typical of washed-curd cheese types, such as Maasdam (Lamichhane et
244 al., 2018a). No significant effect of treatment was observed for the mean value of pH during
245 ripening.

246 *3.3. Growth and viability of Lactococcus lactis, Lactobacillus helveticus and NSLAB*

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

249 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
250 and potentially lysis of some *Lactococcus lactis* during ripening. Moreover, the count of
251 *Lactococcus lactis* was significantly higher ($P < 0.05$) in noWR cheeses than other cheeses,
252 suggesting that the death and possibly lysis of *Lactococcus lactis* was accelerated by the
253 warm room ripening.

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

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

266 3.4. Proteolysis

267 3.4.1. Nitrogen soluble at pH 4.6 (% of total nitrogen)

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

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

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

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

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

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

300 3.4.2. Urea-polyacrylamide gel electrophoresis

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

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

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

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

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

329 3.5. *Insoluble calcium contents of cheeses*

330 The mean level of insoluble calcium (percentage of total calcium) decreased significantly (P
331 < 0.001 , Table 3) during ripening (Fig. 3), especially at the early stage of ripening, from
332 $\sim 75\%$ at 1 d to $\sim 66\%$ at 20 d. After 20 d of ripening, the rate of decrease in the level of
333 insoluble calcium was slower than at the early stages of ripening, which is in agreement with
334 the previous studies in different cheese types (O'Mahony et al., 2005; Lee, Johnson,
335 Govindasamy-Lucey, Jaeggi, & Lucey, 2010).

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

1 346 pepstatin A to the curd/whey mixture during cheese manufacture had no significant effect on
2 347 insoluble calcium content.

3 348 3.6. Fracture properties

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5 349 The fracture properties of experimental cheeses were studied at two different temperatures,
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8 350 i.e., 4 °C or 23 °C (Fig. 4). The stress at fracture (σ_f) and strain at fracture (ϵ_f) were
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11 351 significantly influenced by treatment and ripening time (Table 3).

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13 352 Fracture stress (σ_f), the force required to cause fracture of cheese, represents the strength or
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16 353 rigidity of the cheese matrix. The σ_f measured at 4 °C or 23 °C decreased significantly (Fig.
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19 354 4a-b; Table 3) in all cheeses over maturation. However, the σ_f was significantly higher ($P <$
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21 355 0.05) in PepA, noWR and CC cheeses compared to control cheeses. A lower σ_f in the control
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23 356 cheeses compared to other experimental cheese types was attributed to higher levels of
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26 357 protein breakdown in the control compared to PepA, noWR and CC cheeses (Fig. 1b). A
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28 358 significant negative correlation (Table 4) between pH 4.6-SN (% TN) and σ_f was observed for
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31 359 the experimental cheeses, which is in agreement with previous studies on Cheddar cheese
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33 360 (McCarthy, Wilkinson, Kelly, & Guinee, 2016). Moreover, the σ_f value was significantly
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36 361 positively (Table 4) correlated with intact α_{S1} -casein. Intact β -casein level was also
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38 362 significantly positively correlated with the value of σ_f ; however, the correlation coefficient (r)
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41 363 value was lower for intact β -casein (Table 4) as compared to intact α_{S1} -casein. This suggests
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43 364 that the intact α_{S1} -casein is the principle load-bearing protein within the semi-hard cheese
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46 365 matrix. No significant correlation was found between the σ_f and insoluble calcium content
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49 366 (Table 4), indicating that the extent of solubilization of calcium after 20 d of ripening had no
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52 367 pronounced influence on the strength of the cheese matrix.

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55 368 Fracture strain (ϵ_f) represents the shortness or brittleness of cheese texture; cheeses with a
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58 369 lower fracture strain value are susceptible to fracture at small deformation (Grappin et al.,
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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 Phe₂₃-Phe₂₄ 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

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

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

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

1 420 caseins have different hydrophilic and hydrophobic blocks. For example, α_{S1} -casein has a
2 421 hydrophilic region between strong hydrophobic regions, whereas the β -casein has a
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4 422 hydrophilic and a hydrophobic region at N and C termini, respectively (Lucey et al., 2003).
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7 423 Therefore, it is likely that the specific hydrolysis of caseins during ripening may alter their
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9 424 molecular interactions within cheese matrix which in turn may influence the texture,
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11 425 rheological and fracture behaviour of cheese. For example, Bogenrief and Olson (1995)
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13 426 observed a degree of melt of Cheddar cheese which was more closely related to the extent of
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15 427 β -CN hydrolysis than the hydrolysis of α_{S1} -CN.
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19 428 Overall, the fracture behaviour of cheese can be modulated by specific hydrolysis of casein,
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21 429 modulation of colloidal calcium associated with casein, or both. Such knowledge is
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23 430 particularly important for designing cheese with desired texture profiles or for designing
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25 431 cheese texture suitable for withstanding increased gas pressures during ripening in some eye-
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27 432 type cheeses, which may help to reduce the incidence of undesirable splits and cracks (Daly
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29 433 et al., 2010). Studies have reported that the occurrence of cracks within the cheese matrix is
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31 434 higher for cheeses with lower ϵ_f (short or brittle texture) (Grappin et al., 1993; Rehn et al.,
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33 435 2011). However, it should be noted that unsuitable cheese texture is one possible contributing
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35 436 factor amongst other factors for the development of undesirable splits or cracks, such as; rate
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37 437 and extent of gas production and its behavior (e.g., solubility and diffusivity) within the
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39 438 cheese matrix; late gas production; and the presence of micro-defects within the cheese
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41 439 matrix (Daly et al., 2010).
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49 440 The σ_f of cheeses measured at 4 °C (Fig. 4a) was considerably higher as compared to same
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51 441 cheeses measured at 23 °C (Fig. 4b) at all stages of ripening, which is attributed to the
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53 442 temperature-induced changes on the components of cheese and their interactions
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55 443 (Lamichhane et al., 2018b). At low temperature (~4 °C), more than half of the milk fat
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57 444 present within the cheese matrix is in a crystallized form, and acts as a reinforcing filler,
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445 contributing to the elastic texture of cheese (Lopez, Briard-Bion, Camier, & Gassi, 2006;
446 Lamichhane et al., 2018b). However, the test temperature (4 °C or 23 °C) had no pronounced
447 effect on the ϵ_f of cheeses at all stages of ripening.

448 3.7. *Microstructure*

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

460 The microstructure of the cheeses (at 90 d ripening) was also visualized using CLSM
461 (Supplementary Fig. 2). In agreement with the previous studies (Lopez, Camier, & Gassi,
462 2007), non-globular, coalesced and aggregated fat globules were observed within the cheese
463 matrix, which is attributed to the aggregation, coalescence, and disruption of the fat globules
464 due to the various cheese manufacture steps, such as cooking and pressing (Lopez et al.,
465 2007). The microstructures of all experimental cheeses were visually similar.

466 4. **Conclusions**

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

1 469 inhibitor i.e., pepstatin A, to the curd/whey mixture during cheese manufacture, substitution
2 470 of FPBC with FPCC or modulating ripening temperature altered the hydrolysis patterns of the
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4 471 caseins during ripening. Moreover, solubilization of colloidal calcium was also observed in
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7 472 all cheeses during ripening.
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10 473 The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher
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12 474 stress at fracture) in cheeses with lower levels of proteolysis or higher levels of intact caseins,
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14 475 primarily α_{S1} -casein. However, contrary to expectation, shortness or brittleness (as indicated
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17 476 by lower strain at fracture) of cheese texture was negatively associated particularly with the
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20 477 level of intact β -casein and also with insoluble calcium content.
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23 478 The results from this study suggest that modulation of hydrolysis of α_{S1} -casein is an effective
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25 479 means for maintaining the strength of the cheese matrix during ripening. This could be
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27 480 achieved by inhibition of residual chymosin activity, substitution of FPBC with FPCC or
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30 481 modulating ripening temperature. However, shortness or brittleness of cheese texture could
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32 482 potentially be altered by maintaining higher levels of intact β -casein or insoluble calcium
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34 483 content or both within the cheese matrix. Shortness or brittleness of cheese has previously
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37 484 been associated with undesirable slits or cracks. Therefore, the role of intact β -casein or
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40 485 insoluble calcium content on fracture behaviour, especially fracture strain, merits further
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42 486 research.
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654 **Figure legends**

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3 655 **Fig. 1.** Age-related changes in the (a) pH and (b) level of nitrogen soluble at pH 4.6,
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5 656 expressed as percentage of total nitrogen, pH 4.6-SN (% TN). Data are the mean of data from
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8 657 three replicate trials; [Error bars represent standard error of mean.](#) Experimental cheese
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10 658 variants were Control (control cheeses), noWR (cheeses without warm-room ripening), CC
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12 659 (cheeses made from fermentation-produced camel chymosin as a coagulant), and PepA
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15 660 (cheeses containing chymosin inhibitor, i.e., pepstatin A).

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18 661 **Fig. 2.** (a) Urea-polyacrylamide gel electrophoretograms of semi-hard cheeses after 1, 20, 48
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20 662 or 90 d. Sodium caseinate (lane NaCn) was included as an intact casein control. Protein bands
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22 663 were identified according to McCarthy et al. (2017): 1, β -casein(f106–209) (γ 2); 2, β -
23 664 casein(f29–209) (γ 1); 3, β -casein(f108–209) (γ 3); 4, β -casein; 5, β -casein(f1–192); 6, α _{S1}-
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25 665 casein; 7, α _{S1}-casein(f102–199); 8, α _{S1}-casein(f24–199). Level of (b) intact α _{S1}-casein and (c)
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27 666 intact β -casein as a percentage of the level at 1 d. [Error bars represent standard error of mean.](#)
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30 667 Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-
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33 668 room ripening), CC (cheeses made from fermentation-produced camel chymosin as a
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36 669 coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

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40 670 **Fig. 3.** Changes in the percentage insoluble Ca (expressed as a percentage of total cheese Ca)
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42 671 as a function of ripening time in semi-hard cheeses. Data are the mean of data from three
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45 672 replicate trials and error bars represent standard error of mean. Experimental cheese variants
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48 673 were Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses
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50 674 made from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses
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52 675 containing chymosin inhibitor, i.e., pepstatin A).

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55 676 **Fig. 4.** Changes in (a-b) fracture stress (σ_f , n = 2) and (c-d) fracture strain (ϵ_f , n = 3),
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58 677 measured at 4 °C (closed symbols) and 23 °C (open symbols), in semi-hard cheese during
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1 678 | ripening. [Error bars represent standard error of mean.](#) Experimental cheese variants were
2 679 Control (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made
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4 680 from fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing
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7 681 chymosin inhibitor, i.e., pepstatin A).
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10 682 **Fig. 5.** Selected cryo-SEM micrographs of (a, e) Control, (b, f) noWR, (c, g) CC, and (d, h)
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12 683 PepA cheeses after 90 d of ripening. Experimental cheese variants were Control (control
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14 684 cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from
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16 685 fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing
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18 686 chymosin inhibitor, i.e., pepstatin A). P = protein matrix, F = fat globules, short arrows =
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20 687 spherical imprints in the protein matrix left by fat globules that were removed during sample
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22 688 preparation, and long arrows = remnant fat from globules partially removed during sample
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25 689 preparation.
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1 **Microstructure and fracture properties of semi-hard cheese: Differentiating the effects**
2 **of primary proteolysis and calcium solubilization**

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11 Abstract

12 The individual roles of hydrolysis of α_{S1} - and β -caseins, and calcium solubilization on the
13 fracture properties of semi-hard cheeses, such as Maasdam and other eye-type cheeses,
14 remain unclear. In this study, the hydrolysis patterns of casein were selectively altered by
15 adding a chymosin inhibitor to the curd/whey mixture during cheese manufacture, by
16 substituting fermentation-produced bovine chymosin (FPBC) with fermentation-produced
17 camel chymosin (FPCC), or by modulating ripening temperature. Moreover, the level of
18 insoluble calcium during ripening was quantified in all cheeses. Addition of a chymosin
19 inhibitor, substitution of FPBC with FPCC, or ripening of cheeses at a consistent low
20 temperature (8 °C) decreased the hydrolysis of α_{S1} -casein by ~95%, ~45%, or ~30%,
21 respectively, after 90 d of ripening, whereas ~35% of β -casein was hydrolysed in that time
22 for all cheeses, except for those ripened at a lower temperature (~17%). The proportion of
23 insoluble calcium as a percentage of total calcium decreased significantly from ~75% to
24 ~60% between 1 and 90 d. The rigidity or strength of the cheese matrix was found to be
25 higher (as indicated by higher fracture stress) in cheeses with lower levels of proteolysis or
26 higher levels of intact caseins, primarily α_{S1} -casein. However, contrary to the expectation that
27 shortness of cheese texture is associated with α_{S1} -casein hydrolysis, fracture strain was
28 significantly positively correlated with the level of intact β -casein and insoluble calcium
29 content, indicating that the cheeses with low levels of intact β -casein or insoluble calcium
30 content were more likely to be shorter in texture (i.e., lower fracture strain). Overall, this
31 study suggests that the fracture properties of cheese can be modified by selective hydrolysis
32 of caseins, altering the level of insoluble calcium or both. Such approaches could be applied
33 to design cheese with specific properties.

34 Keywords: Cheese; Proteolysis; Insoluble calcium; Fracture properties; Microstructure; Split
35 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

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

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

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

88 2. Materials and methods

89 2.1. Milk supply and cheese manufacture

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90 Raw milk was obtained from the Teagasc Animal and Grassland Research and Innovation
91 Centre, Moorepark, Ireland. Raw milk was first separated into skim milk and cream using
92 bench top centrifugal separator. Using skim milk and cream, cheese milks were standardized
93 to a protein to fat ratio of 1.10:1.00, with an average protein and fat content of 3.52 % (w/w)
94 and 3.21 % (w/w), respectively. The standardized cheese-milks were then pasteurized at 72
95 °C for 15 sec (MicroThermics, USA) and stored at 4 °C overnight prior to cheese
96 manufacture.

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97 Washed-curd brine-salted semi-hard cheeses were manufactured in triplicate trials over a 3
98 month period. Standardized and pasteurized cheese milks were placed into jacketed cheese
99 vats (Pierre Guerin Technologies, Niort, France) with each vat containing 11 kg cheese milk,
100 for each replication. Each vat contained automated variable speed cutting and stirring
101 equipment. All cheese milks were inoculated at 32 °C with frozen direct vat inoculation
102 cultures: consisting of (1) R-604 (180 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland),
103 containing *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*; and (2) LH-B02
104 (9 mg/kg milk; Chr. Hansen Ltd., Cork, Ireland), containing *Lactobacillus helveticus*.
105 Propionic acid bacteria were not inoculated into the cheese milks to avoid subsequent eye-
106 formation during ripening of cheese which would not permit measurement of texture
107 parameters.

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

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

129 Whey was drained when the curd pH reached 6.35, and the curds were collected into moulds
130 and pressed vertically under increasing pressure from 40 to 75 kPa for ~4.5 hours. When the
131 pH of the cheese curds reached ~5.50, the cheese wheels (~600 g each) were transferred to a
132 saturated brine solution (23%, w/w, NaCl, 0.56%, w/w, CaCl₂, and pH 5.2) for 7.5 h at 8 °C.

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

138 2.2. *Milk and cheese composition*

139 The composition of raw and pasteurized (72 °C for 15 s) cheese milks were analyzed as
140 described by Lamichhane, Kelly, and Sheehan (2018a). Grated cheese samples were analyzed
141 at 20 d of ripening in duplicate for moisture, fat, protein and salt as described by Hickey et al.
142 (2018b). Cheese pH was measured at 1, 20, 48 and 90 d as described by Sheehan, Fenelon,
143 Wilkinson, and McSweeney (2007).

144 2.3. *Enumeration of starter and nonstarter lactic acid bacteria*

145 Samples were removed from cheese wheels using a cheese trier at 1, 20, 48 and 90 d of
146 ripening. Cheese samples were prepared as described by Lamichhane et al. (2018c). Viable
147 *Lactococcus lactis* cells were enumerated on M17 (Difco Laboratories; Detroit, MI) medium,
148 supplemented with 0.5% (w/v) lactose, after aerobic incubation at 25 °C for 3 d (Ruggirello
149 et al., 2018). Total numbers of *Lactobacillus helveticus* cells were enumerated on de Man,
150 Rogosa, and Sharpe agar (BD, Oxford, UK) at pH 5.4 after anaerobic incubation for 3 d at 42
151 °C (Lamichhane et al., 2018c). Nonstarter lactic acid bacteria (NSLAB) cells were
152 enumerated on *Lactobacillus* selection agar (BD), with an overlay, after aerobic incubation
153 for 5 d at 30 °C (Lamichhane et al., 2018c).

154 2.4. *Proteolysis*

155 2.4.1. *pH 4.6-soluble nitrogen (% of total nitrogen)*

156 The levels of nitrogen soluble (expressed as % of total nitrogen) at pH 4.6 was measured after
157 1, 20, 48, and 90 d as described by Fenelon and Guinee (2000).

158 2.4.2. *Urea-polyacrylamide gel electrophoresis*

159 Urea-polyacrylamide gel electrophoresis (PAGE) of the cheeses at 1, 20, 48 and 90 d was
160 performed, in duplicate, on a Protean II xi vertical slab gel unit (Biorad Laboratories Ltd.,
161 Watford, Herts, UK), as described by Sheehan and Guinee (2004a). Briefly, grated cheese
162 samples (equivalent to 4 mg protein) were dissolved in 1 mL sample buffer, incubated at 55
163 °C for 10 min and each sample was loaded at a level of 12 µL per well. Sodium caseinate
164 powder (Kerry Ingredients, Listowel) was used as an intact casein control. The samples ran
165 initially through the stacking gel at 280 V and then through the separating gel at 300 V. The
166 resulting gels were stained and scanned as described by McCarthy, Wilkinson, and Guinee
167 (2017). Densitometry analysis was performed on the scanned images using image analysis
168 software i.e., ImageJ (NIH, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>). Eight major
169 bands corresponding to caseins or its breakdown products were used for calculation: 1, β-
170 casein(f106–209) (γ2); 2, β-casein(f29–209) (γ1); 3, β-casein(f108–209) (γ3); 4, β-casein; 5,
171 β-casein(f1–192); 6, α_{S1}-casein; 7, α_{S1}-casein(f102–199); 8, α_{S1}-casein(f24–199). The area of
172 each protein band was expressed as a percentage of total band area of these eight major
173 bands. Levels of intact α_{S1}-casein and β-casein over ripening were expressed as a percentage
174 of level at 1 d.

175 2.5. *Determination of total and insoluble calcium content*

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

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

180 2.6. Fracture properties

181 Eight to 10 cylindrical samples (height 15 mm and diameter 12 mm) of each cheese were
182 removed, using a borer and a wire cutter, at 20, 48 and 90 d of ripening. The cheese samples
183 were wrapped in tin foil; half of the cylindrical cheese samples were stored at 4 °C and the
184 remainder was stored at 23 °C for at least 4 hours. Cheese samples (at 4 °C or 23 °C) were
185 compressed at a rate of 60 mm/min until fracture. True stress (σ ; Equation 1) and Hencky
186 strain (ε_H ; Equation 2) were calculated, assuming a constant volume deformation (Rehn et al.,
187 2011):

$$188 \quad \sigma = \frac{FH_t}{A_0H_0} \quad (1)$$

$$189 \quad \varepsilon_H = \left| \ln \frac{H_t}{H_0} \right| \quad (2)$$

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

194 2.7. Visualization of cheese microstructure

195 Cheese microstructure was observed using cryogenic-scanning electron microscopy (cryo-
196 SEM). This was conducted using an SEM system (SEM-Zeiss Supra 40VP field emission,
197 Carl Zeiss AG, Darmstadt, Germany) with a cryogenic transfer system attached (Gatan Alto
198 2500, Gatan UK). Fresh cheese samples (after 90 d of ripening) were taken from the middle
199 of each experimental cheese wheel and rapidly immersed into a liquid nitrogen slush (-200

200 °C) in a cryo-preparation chamber. The samples were transferred under vacuum into the high
201 vacuum cryo-preparation chamber at -185 °C, etched at -95 °C over a period of 15 min,
202 sputter-coated at -125 °C and finally transferred onto the SEM cold stage at -125 °C. Cryo-
203 SEM images were acquired at -125 °C.

204 The microstructure of cheese samples was also visualised using confocal laser scanning
205 microscopy (Leica TCS SP5, Leica Microsystems, Baden-Württemberg, Germany).

206 Rectangular cheese samples (5 mm × 5 mm × 2 mm) were removed from cheeses using a
207 sharp scalpel. Solutions of the protein specific dye Fast Green (Sigma Aldrich) and fat
208 specific dye Nile Red (Sigma Aldrich) were prepared at a concentration of 0.01% (w/v) in
209 1,2-propanediol (Sigma Aldrich) and deionized water respectively, which were then mixed at
210 a ratio of 3:1. The prepared dye mixture (40 µL) was applied to the surface of cheese
211 samples; a cover slip was gently placed on top and the sample was held at 4 °C for 10 min
212 prior to imaging. The protein and fat phases of the cheese samples were visualised by
213 exciting the Fast Green dye (using a He-Ne laser; excitation wavelength of 633 nm and
214 emission wavelength range of 650-700 nm) and Nile Red dye (using an Argon laser;
215 excitation wavelength of 488 nm and emission wavelength range of 500-580 nm) respectively
216 as described by Abhyankar, Mulvihill, and Auty (2014). All images were acquired using an
217 oil immersion objective with a numerical aperture of 1.4 and a magnification of 63× (Leica
218 Microsystems, Baden-Württemberg, Germany).

219 2.8. Statistical analysis

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

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

231 **3. Results and discussion**

232 *3.1. Milk and cheese composition*

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

240 *3.2. pH*

241 The pH of all experimental cheeses increased significantly ($P < 0.001$; Table 3) during
242 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
243 consistent with that typical of washed-curd cheese types, such as Maasdam (Lamichhane et
244 al., 2018a). No significant effect of treatment was observed for the mean value of pH during
245 ripening.

246 *3.3. Growth and viability of Lactococcus lactis, Lactobacillus helveticus and NSLAB*

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

249 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
250 and potentially lysis of some *Lactococcus lactis* during ripening. Moreover, the count of
251 *Lactococcus lactis* was significantly higher ($P < 0.05$) in noWR cheeses than other cheeses,
252 suggesting that the death and possibly lysis of *Lactococcus lactis* was accelerated by the
253 warm room ripening.

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

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

266 3.4. Proteolysis

267 3.4.1. Nitrogen soluble at pH 4.6 (% of total nitrogen)

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

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

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

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

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

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

300 3.4.2. Urea-polyacrylamide gel electrophoresis

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

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

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

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

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2 322 influenced the hydrolysis of β -casein in agreement with the previous studies (O'Mahony et
3 323 al., 2005; Bansal et al., 2009). However, the extent of breakdown was relatively lower in
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5 324 noWR cheeses (i.e., less than 20% of levels at 1 d) than other cheeses, suggesting that warm
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7 325 room ripening accelerates the degradation of β -casein. Overall, these results suggest that the
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9 326 various hydrolysis patterns of casein can be achieved by using different coagulant types,
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11 327 modulating ripening temperature or inhibiting residual chymosin activity, although inhibition
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13 328 of the latter using pepstatin A is obviously not commercially viable.
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17 329 3.5. *Insoluble calcium contents of cheeses*

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20 330 The mean level of insoluble calcium (percentage of total calcium) decreased significantly (P
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22 331 < 0.001 , Table 3) during ripening (Fig. 3), especially at the early stage of ripening, from
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24 332 $\sim 75\%$ at 1 d to $\sim 66\%$ at 20 d. After 20 d of ripening, the rate of decrease in the level of
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26 333 insoluble calcium was slower than at the early stages of ripening, which is in agreement with
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28 334 the previous studies in different cheese types (O'Mahony et al., 2005; Lee, Johnson,
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30 335 Govindasamy-Lucey, Jaeggi, & Lucey, 2010).
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35 336 The effect of warm-room ripening on solubilization of colloidal calcium in brine-salted
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37 337 cheese varieties has not previously been studied. Therefore, the rate of calcium solubilization
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39 338 was compared between cheeses subjected to warm room ripening (control cheeses) and
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41 339 without warm room ripening (noWR cheeses). Interestingly, the mean insoluble calcium
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43 340 content of noWR cheeses was $\sim 3\%$ higher than that of the control cheese after 48 d of (after
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45 341 warm room ripening); however, the difference observed was not statistically significant,
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47 342 suggesting that, at best, the warm room ripening had only a minor effect on the solubilization
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49 343 of calcium. Hydrolysis of β -casein is known to release phosphopeptides (Gagnaire, Mollé,
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51 344 Herrouin, & Léonil, 2001), which could contribute to decreases in the level of casein-bound
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53 345 calcium. As expected, substitution of FPBC with FPCC as a coagulant or addition of
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1 346 pepstatin A to the curd/whey mixture during cheese manufacture had no significant effect on
2 347 insoluble calcium content.

3 348 3.6. Fracture properties

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5 349 The fracture properties of experimental cheeses were studied at two different temperatures,
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8 350 i.e., 4 °C or 23 °C (Fig. 4). The stress at fracture (σ_f) and strain at fracture (ϵ_f) were
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11 351 significantly influenced by treatment and ripening time (Table 3).

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13 352 Fracture stress (σ_f), the force required to cause fracture of cheese, represents the strength or
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16 353 rigidity of the cheese matrix. The σ_f measured at 4 °C or 23 °C decreased significantly (Fig.
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19 354 4a-b; Table 3) in all cheeses over maturation. However, the σ_f was significantly higher ($P <$
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22 355 0.05) in PepA, noWR and CC cheeses compared to control cheeses. A lower σ_f in the control
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25 356 cheeses compared to other experimental cheese types was attributed to higher levels of
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28 357 protein breakdown in the control compared to PepA, noWR and CC cheeses (Fig. 1b). A
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31 358 significant negative correlation (Table 4) between pH 4.6-SN (% TN) and σ_f was observed for
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33 359 the experimental cheeses, which is in agreement with previous studies on Cheddar cheese
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36 360 (McCarthy, Wilkinson, Kelly, & Guinee, 2016). Moreover, the σ_f value was significantly
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38 361 positively (Table 4) correlated with intact α_{S1} -casein. Intact β -casein level was also
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41 362 significantly positively correlated with the value of σ_f ; however, the correlation coefficient (r)
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43 363 value was lower for intact β -casein (Table 4) as compared to intact α_{S1} -casein. This suggests
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45 364 that the intact α_{S1} -casein is the principle load-bearing protein within the semi-hard cheese
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48 365 matrix. No significant correlation was found between the σ_f and insoluble calcium content
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51 366 (Table 4), indicating that the extent of solubilization of calcium after 20 d of ripening had no
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53 367 pronounced influence on the strength of the cheese matrix.

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55 368 Fracture strain (ϵ_f) represents the shortness or brittleness of cheese texture; cheeses with a
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58 369 lower fracture strain value are susceptible to fracture at small deformation (Grappin et al.,
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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 Phe₂₃-Phe₂₄ 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

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

406 In the current study, insoluble calcium (expressed as a percentage of total calcium) and intact
407 β -casein were significantly positively correlated with ϵ_f (Table 4). Furthermore, levels of
408 intact β -casein (Fig. 2c) and insoluble calcium (Fig. 3) were on average ~15% and ~3%
409 higher, respectively, in noWR cheeses than in the other cheeses after 48 d of ripening. This
410 suggests that the breakdown of intact β -casein, solubilization of colloidal calcium during
411 ripening, or both may contribute to a shorter texture (i.e., lower ϵ_f) observed in control, CC
412 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.

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

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

428 Overall, the fracture behaviour of cheese can be modulated by specific hydrolysis of casein,
429 modulation of colloidal calcium associated with casein, or both. Such knowledge is
430 particularly important for designing cheese with desired texture profiles or for designing
431 cheese texture suitable for withstanding increased gas pressures during ripening in some eye-
432 type cheeses, which may help to reduce the incidence of undesirable splits and cracks (Daly
433 et al., 2010). Studies have reported that the occurrence of cracks within the cheese matrix is
434 higher for cheeses with lower ϵ_f (short or brittle texture) (Grappin et al., 1993; Rehn et al.,
435 2011). However, it should be noted that unsuitable cheese texture is one possible contributing
436 factor amongst other factors for the development of undesirable splits or cracks, such as; rate
437 and extent of gas production and its behavior (e.g., solubility and diffusivity) within the
438 cheese matrix; late gas production; and the presence of micro-defects within the cheese
439 matrix (Daly et al., 2010).

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

445 contributing to the elastic texture of cheese (Lopez, Briard-Bion, Camier, & Gassi, 2006;
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2 446 Lamichhane et al., 2018b). However, the test temperature (4 °C or 23 °C) had no pronounced
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5 447 effect on the ϵ_f of cheeses at all stages of ripening.
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7 448 3.7. *Microstructure*

10 449 The microstructure of cheese (at 90 d of ripening) observed by cryo-SEM is shown in Fig. 5.
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13 450 The microstructure of the control cheese is clearly different from that of the other
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15 451 experimental cheese types; the microstructure observed for the control cheese was more open
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18 452 than that of the other experimental cheeses. The open structure may be attributed to
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20 453 significantly higher levels of proteolysis in the control cheeses compared to the other cheese
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23 454 types. For other experimental cheeses, the microstructure looks visually similar. During
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25 455 proteolysis, the intact caseins, which are responsible for network formation, breakdown into
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28 456 small and medium size peptides and free amino acids and these peptides and amino acids are
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30 457 released into the serum fraction of the cheese (Sousa, Ardö, & McSweeney, 2001). Soodam,
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33 458 Ong, Powell, Kentish, and Gras (2015) also observed a less open structure of cheese with low
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35 459 levels of primary proteolysis than in cheeses with high levels.
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38 460 The microstructure of the cheeses (at 90 d ripening) was also visualized using CLSM
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41 461 (Supplementary Fig. 2). In agreement with the previous studies (Lopez, Camier, & Gassi,
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43 462 2007), non-globular, coalesced and aggregated fat globules were observed within the cheese
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45 463 matrix, which is attributed to the aggregation, coalescence, and disruption of the fat globules
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48 464 due to the various cheese manufacture steps, such as cooking and pressing (Lopez et al.,
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50 465 2007). The microstructures of all experimental cheeses were visually similar.
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53 466 4. **Conclusions**

56 467 The roles of primary proteolysis and calcium solubilization on the fracture properties of
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59 468 washed-curd brine-salted semi-hard cheese were investigated. Addition of a chymosin
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1 469 inhibitor i.e., pepstatin A, to the curd/whey mixture during cheese manufacture, substitution
2 470 of FPBC with FPCC or modulating ripening temperature altered the hydrolysis patterns of the
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4 471 caseins during ripening. Moreover, solubilization of colloidal calcium was also observed in
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7 472 all cheeses during ripening.
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10 473 The rigidity or strength of the cheese matrix was found to be higher (as indicated by higher
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12 474 stress at fracture) in cheeses with lower levels of proteolysis or higher levels of intact caseins,
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15 475 primarily α_{S1} -casein. However, contrary to expectation, shortness or brittleness (as indicated
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17 476 by lower strain at fracture) of cheese texture was negatively associated particularly with the
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20 477 level of intact β -casein and also with insoluble calcium content.
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23 478 The results from this study suggest that modulation of hydrolysis of α_{S1} -casein is an effective
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25 479 means for maintaining the strength of the cheese matrix during ripening. This could be
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28 480 achieved by inhibition of residual chymosin activity, substitution of FPBC with FPCC or
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30 481 modulating ripening temperature. However, shortness or brittleness of cheese texture could
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32 482 potentially be altered by maintaining higher levels of intact β -casein or insoluble calcium
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35 483 content or both within the cheese matrix. Shortness or brittleness of cheese has previously
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37 484 been associated with undesirable slits or cracks. **Therefore, the role of intact β -casein or**
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40 485 **insoluble calcium content on fracture behaviour, especially fracture strain, merits further**
41
42 486 **research.**
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654 **Figure legends**

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3 655 **Fig. 1.** Age-related changes in the (a) pH and (b) level of nitrogen soluble at pH 4.6,
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5 656 expressed as percentage of total nitrogen, pH 4.6-SN (% TN). Data are the mean of data from
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8 657 three replicate trials. Experimental cheese variants were Control (control cheeses), noWR
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10 658 (cheeses without warm-room ripening), CC (cheeses made from fermentation-produced
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13 659 camel chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e.,
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15 660 pepstatin A).

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18 661 **Fig. 2.** (a) Urea-polyacrylamide gel electrophoretograms of **semi-hard** cheeses after 1, 20, 48
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20 662 or 90 d. Sodium caseinate (lane NaCn) was included as an intact casein control. Protein bands
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23 663 were identified according to McCarthy et al. (2017): 1, β -casein(f106–209) (γ 2); 2, β -
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25 664 casein(f29–209) (γ 1); 3, β -casein(f108–209) (γ 3); 4, β -casein; 5, β -casein(f1–192); 6, α _{S1}-
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27 665 casein; 7, α _{S1}-casein(f102–199); 8, α _{S1}-casein(f24–199). Level of (b) intact α _{S1}-casein and (c)
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30 666 intact β -casein as a percentage of the level at 1 d. Experimental cheese variants were Control
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33 667 (control cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from
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35 668 fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing
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37 669 chymosin inhibitor, i.e., pepstatin A).

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40 670 **Fig. 3.** Changes in the percentage insoluble Ca (expressed as a percentage of total cheese Ca)
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43 671 as a function of ripening time in **semi-hard** cheeses. Data are the mean of data from three
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45 672 replicate trials and error bars show the standard error of mean from 3 replicate trials.
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48 673 Experimental cheese variants were Control (control cheeses), noWR (cheeses without warm-
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50 674 room ripening), CC (cheeses made from fermentation-produced camel chymosin as a
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53 675 coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin A).

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55 676 **Fig. 4.** Changes in (a-b) fracture stress (σ_f , n = 2) and (c-d) fracture strain (ϵ_f , n = 3),
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58 677 measured at 4 °C (closed symbols) and 23 °C (open symbols), in **semi-hard** cheese during
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1 678 ripening. Experimental cheese variants were Control (control cheeses), noWR (cheeses
2 679 without warm-room ripening), CC (cheeses made from fermentation-produced camel
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4 680 chymosin as a coagulant), and PepA (cheeses containing chymosin inhibitor, i.e., pepstatin
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7 681 A).

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10 682 **Fig. 5.** Selected cryo-SEM micrographs of (a, e) Control, (b, f) noWR, (c, g) CC, and (d, h)
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12 683 PepA cheeses after 90 d of ripening. Experimental cheese variants were Control (control
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14 684 cheeses), noWR (cheeses without warm-room ripening), CC (cheeses made from
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16 685 fermentation-produced camel chymosin as a coagulant), and PepA (cheeses containing
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18 686 chymosin inhibitor, i.e., pepstatin A). P = protein matrix, F = fat globules, short arrows =
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21 687 spherical imprints in the protein matrix left by fat globules that were removed during sample
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24 688 preparation, and long arrows = remnant fat from globules partially removed during sample
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27 689 preparation.

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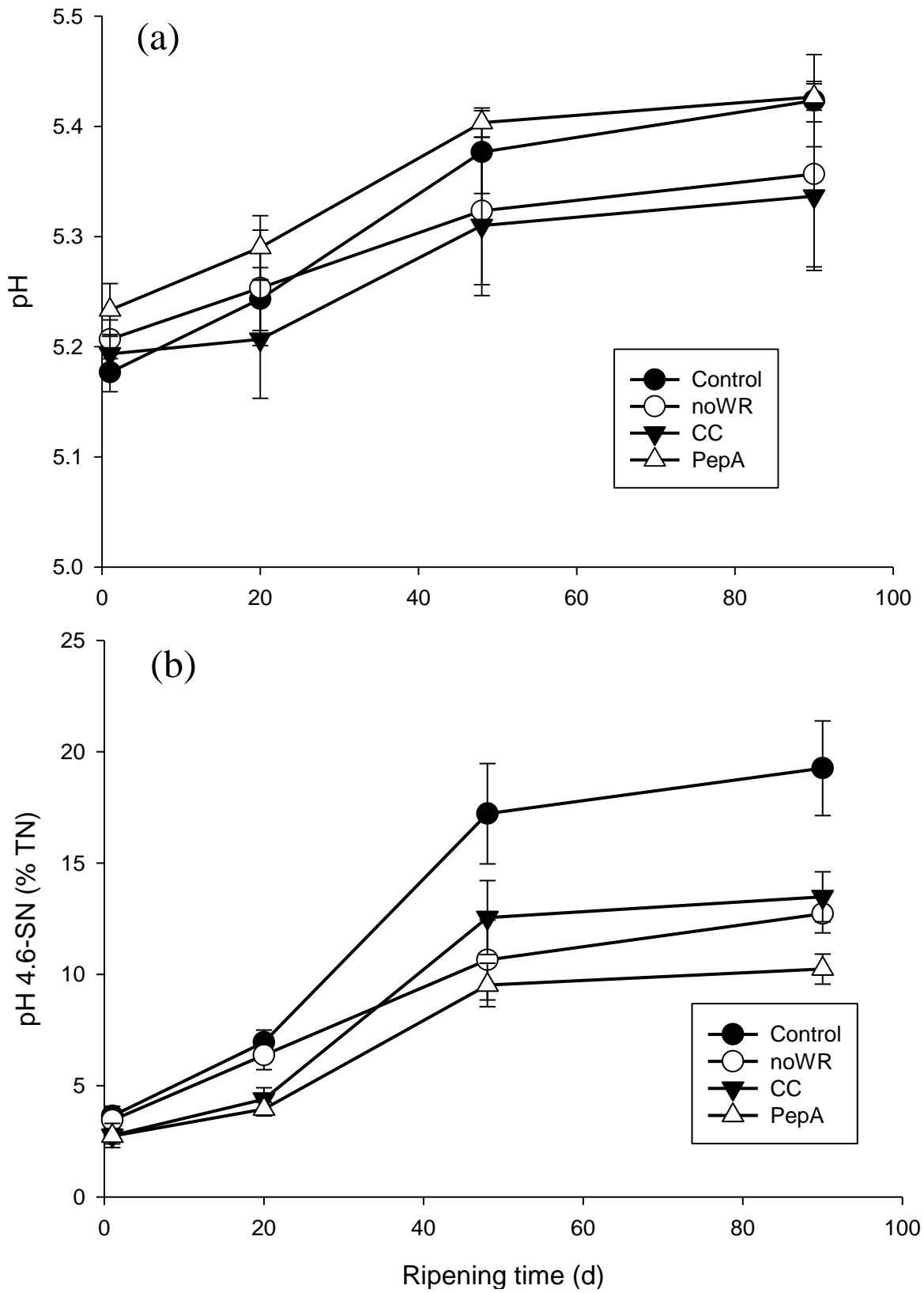


Figure 1.

Figure 2

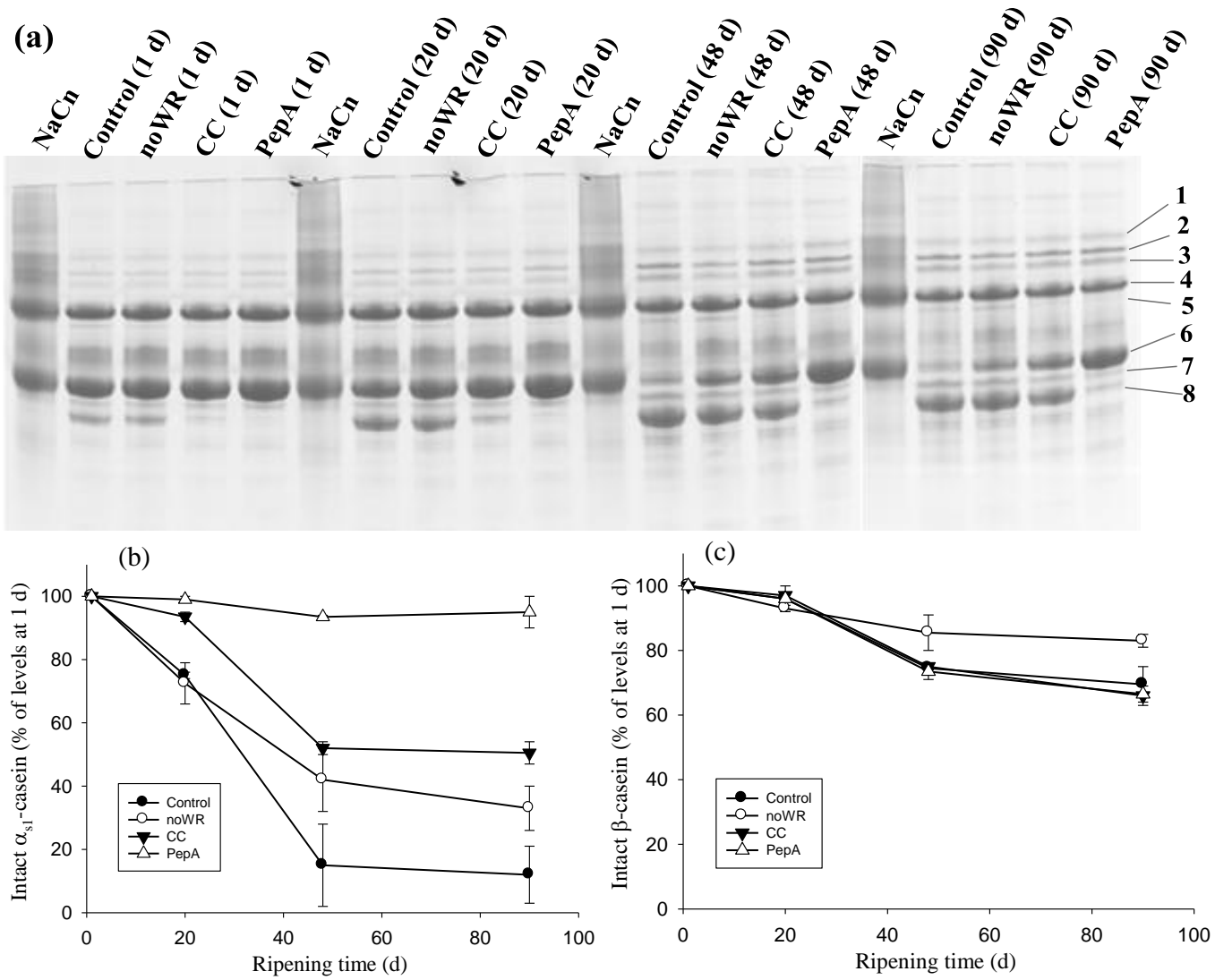


Figure 2.

Figure 3

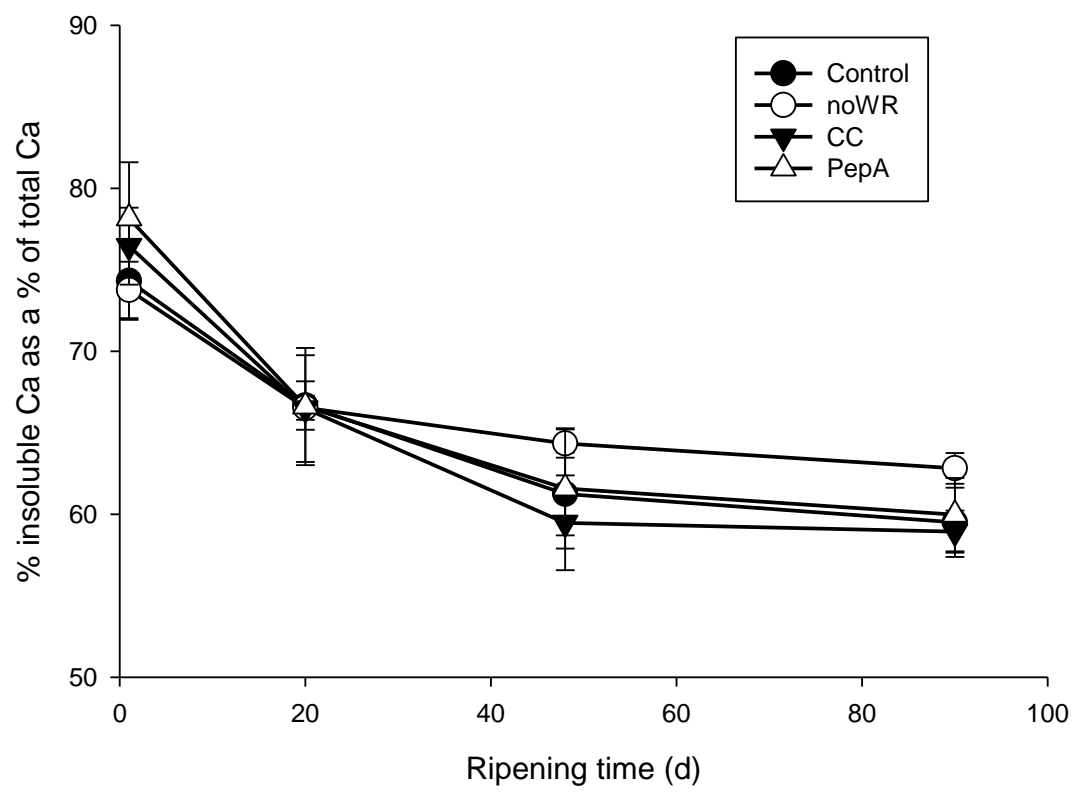


Figure 3.

Figure 4

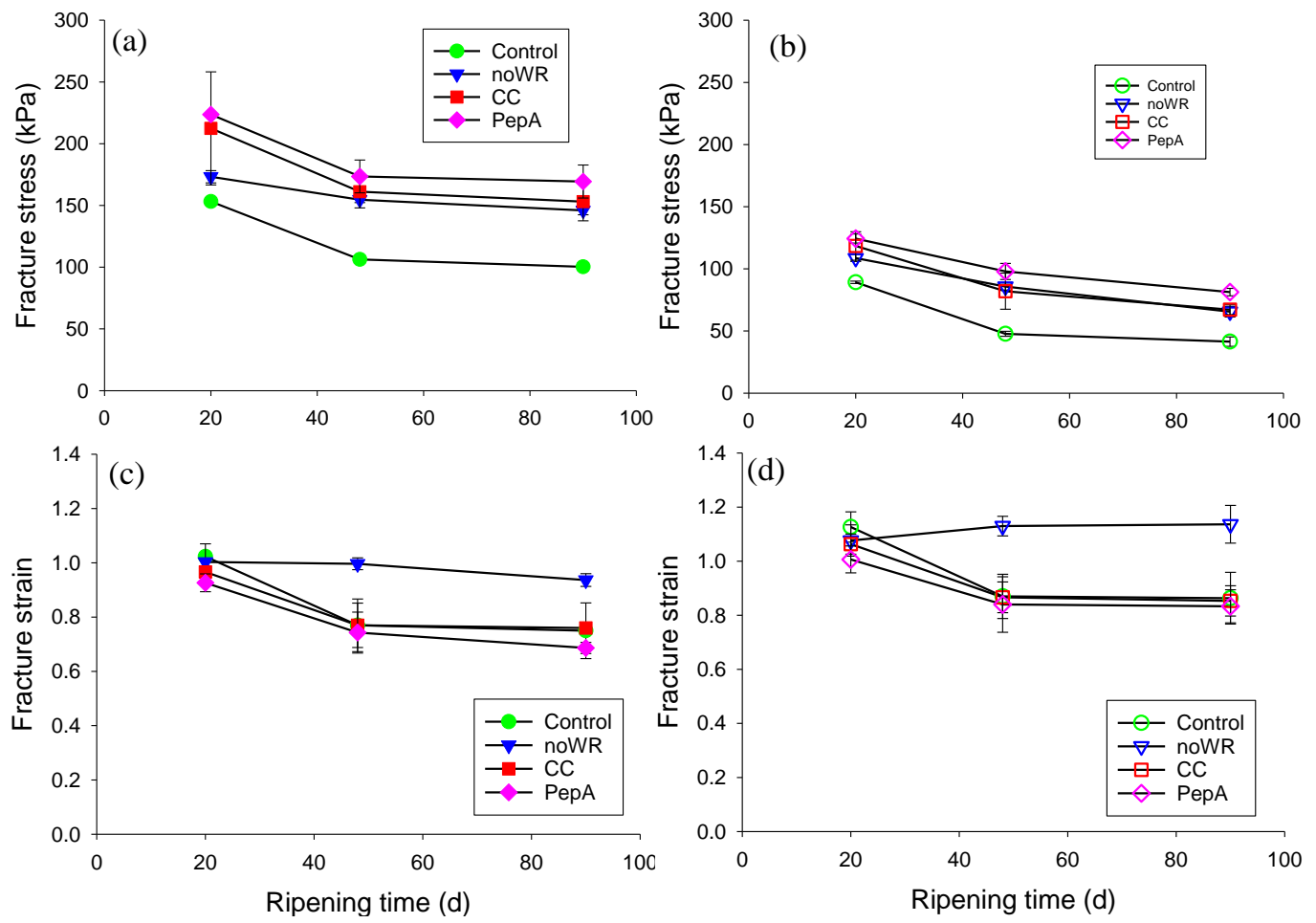


Figure 4.

Figure 5

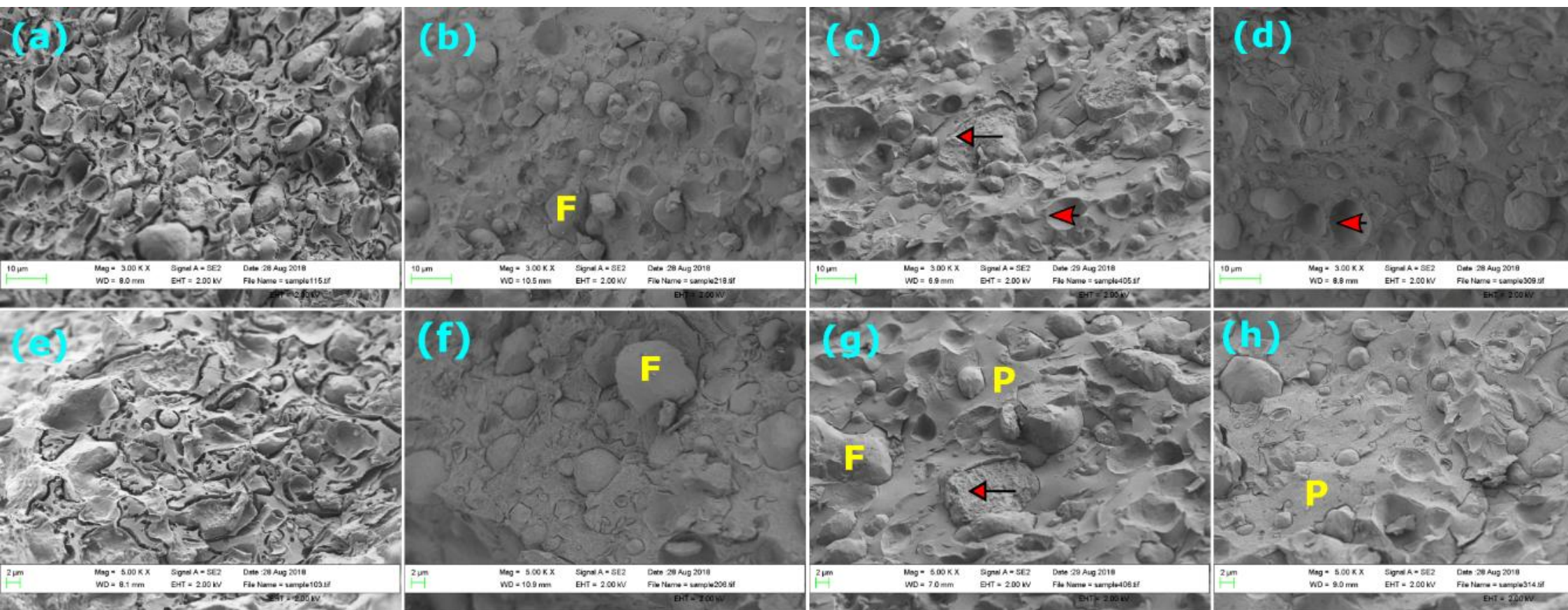


Fig. 5.

Table 1. General overview of the treatments and ripening regimens used in the study¹

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

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

²noWR, 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. Compositional parameters at 20 d and pH at 1 d of ripening in semi-hard cheeses¹

Compositional factors	Cheese types ²				<i>P</i> -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

¹MNFS, 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.

²Values 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. Summary of the effects of treatment, time and their interactions on properties of semi-hard cheeses¹

Parameter	Treatment	Time	Interactive effect (treatment × time)
pH	NS	***	NS
<i>Lactococcus lactis</i> count	**	***	NS
<i>Lactobacillus helveticus</i> 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

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

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

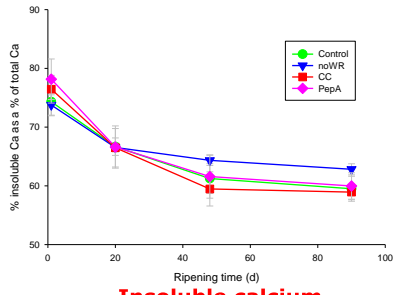
Table. 4. Pearson correlation coefficients between fracture parameters, primary proteolysis, insoluble calcium, and intact β -casein and α_{S1} -casein¹

	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}

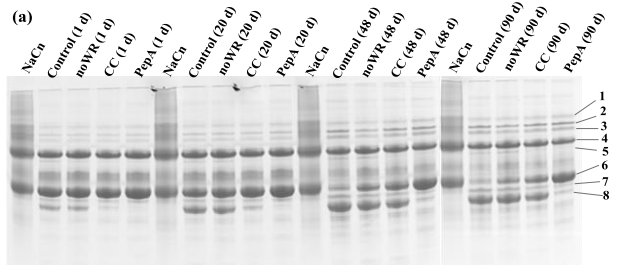
¹ σ_f , stress at fracture; ε_f , strain at fracture; data were obtained from all experimental cheeses over a 90 d of ripening.

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

Graphical abstract



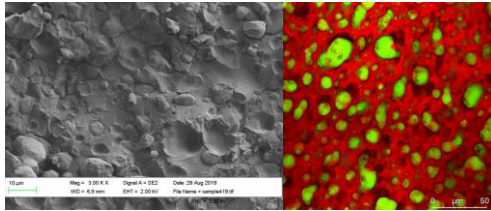
Insoluble calcium



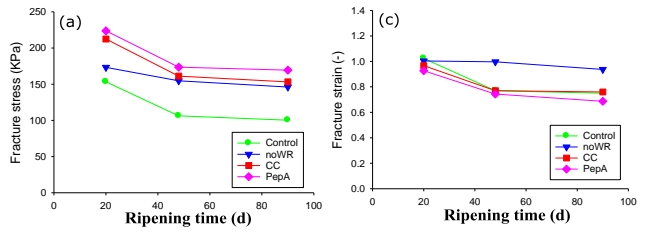
Proteolysis



Cheese



Microstructure



Fracture properties