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1 **Effects of shredding on the functionality, microstructure and**
2 **proteolysis of low-moisture Mozzarella cheese.**

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12 Low-moisture Mozzarella cheese is commonly shredded before packaging, however, the effects
13 of shredding are not fully understood. Industrially produced block and shredded Mozzarella
14 cheese were studied during 8 weeks of storage at 4 °C. Cheese shredded at both 15 d and 8
15 weeks, coated with microcrystalline cellulose and stored in a modified atmosphere (70% N₂ and
16 30% CO₂), had an altered microstructure after 8 weeks compared to vacuum packed block
17 cheese, where the fat was more dispersed. Proteolysis was also higher in shredded samples and
18 a higher level of two bacterial proteases was detected. Despite these differences, the functional
19 properties of the block and shredded samples were similar at 8 weeks. The microstructure and
20 functionality of cheese shredded at 15 d and stored for a further 6 weeks was also similar to
21 cheese shredded after 8 weeks, suggesting there is a flexible time period over which shredding
22 processes can be performed.

23 **Highlights**

- 24 • Block and shredded cheese (with MCC and MAP) were stored at 4 °C for 8 weeks.
- 25 • On storage, microstructure and proteolysis differed in shredded and block cheese.
- 26 • The functionality of shredded and block cheese was similar.
- 27 • Shredding after 2 or 8 weeks gave similar functional properties.

28

29 **Keywords:** processing; confocal microscopy; CLSM; functional properties; cheese shred.

30

31 **1. Introduction**

32 Low-moisture Mozzarella cheese blocks are often shredded for use in processed foods, such as
33 pizza (Banville, Morin, Pouliot, & Britten, 2013). Once shredded, the cheese is usually stored
34 chilled in a modified atmosphere environment consisting of a mixture of carbon dioxide and
35 nitrogen (Eliot, Vuillemand, & Emond, 1998). Anticaking agents, often cellulose based such as
36 microcrystalline cellulose or starch based (Akins, 2002), may also be added to the shredded
37 product to prevent the adhesion of cheese shreds, which leads to undesirable clumping
38 (Elayedath & Barringer, 2002).

39 The effect of storage on the microstructure and functionality of low-moisture Mozzarella cheese
40 blocks has been studied by a range of researchers (Conte, Scrocco, Sinigaglia, & Del Nobile,
41 2007; Guinee, Feeney, Auty, & Fox, 2002; Jana & Mandal, 2011). There are few studies, however,
42 that have focused on the effect of storage on the functionality of shredded Mozzarella cheese
43 (Oberg, Merrill, Brown, & Richardson, 1992; Singh, Kanawjia, Giri, & Khetra, 2015). To date, such
44 studies have focused on stability during storage in microbiological or quality terms or on the
45 growth of microbial populations in a modified atmosphere (Eliot et al., 1998; Sikin, Walkling-
46 Ribeiro, & Rizvi, 2016; Singh et al., 2015). A study examining the industrial shredding process
47 and storage-related changes in the microstructure, proteolysis and functionality of the resultant
48 cheeses would provide new insights into the effects of the shredding process on the properties
49 of Mozzarella cheese.

50 Constraints associated with the age of the cheese commonly dictate an optimum window for
51 shredding. Low-moisture Mozzarella is typically shredded no earlier than two weeks after
52 processing, once the free moisture present in freshly made cheese (the expressible serum) is
53 absorbed into the protein network (Kindstedt, 1995). Shredding before this time, when
54 expressible serum is still present, may lead to increased fines and increased stickiness that can
55 reduce yield (Guo & Kindstedt, 1995). It has also been noted that a high level of moisture (above

56 50%) generally softens the cheese and reduces shreddability (Kindstedt, 1995). Mozzarella also
57 softens with aging (Banville, Morin, Pouliot, & Britten, 2014) and shreddability is reduced for
58 softer cheese, due in part to its tendency to stick to the shredding blade. This softening process
59 is continuous with age, caused by the combined effects of proteolysis (Kindstedt, 1995) and the
60 solubilisation of colloidal calcium phosphate (O'Mahony, McSweeney, & Lucey, 2006; Smith,
61 Hindmarsh, Carr, Golding, & Reid, 2017). A reduction of bound calcium during aging also
62 decreases interactions between casein, leading to a weaker cheese structure (Hassan, Johnson,
63 & Lucey, 2004; Hickey, Auty, Wilkinson, & Sheehan, 2015), which can potentially be monitored
64 by phosphorous NMR.

65 The softening of cheese results from protein breakdown during proteolysis (Tunick et al., 1993).
66 Within Mozzarella, proteolysis is primarily a result of enzymatic activity from starter cultures
67 (Guinee et al., 2002), as well as residual coagulant (Costabel, Pauletti, & Hynes, 2007).
68 Increasing the cooking-stretching temperature above 83 °C leads to less residual coagulant
69 activity, although extended storage of more than four months at 4 °C can lead to similar levels of
70 proteolytic breakdown when cooking-stretching temperatures are varied between 75 °C and 91
71 °C (Costabel et al., 2007), potentially as a result of the levels of residual coagulant activity.

72 Shredding has previously been found to have no impact on the level of proteolysis occurring
73 after 40 days of storage at 4°C, when comparing blocks and shredded Mozzarella (Cortez,
74 Furtado, Gigante, & Kindstedt, 2008). Whilst functionality tests were performed on shredded
75 product in this study, these did not directly compare the functionality of block and shredded
76 products, as the focus was on the effect of shredded cheese pH. Specifically, the pH was adjusted
77 between pH 5.2 and pH 6.1 and found to impact on hardness and meltability, with lower pH
78 leading to a softer cheese that melted more easily (Cortez et al., 2008).

79 The microstructure within a block Mozzarella cheese product typically features protein and fat
80 that are aligned as a result of cooking and stretching during cheesemaking, where the fat

81 droplets appear in channels in the direction of the shear aligned protein network. The structural
82 alignment of protein and fat has also been related to both the meltability and stretchability of
83 Mozzarella cheese (Smith, Carr, Golding, & Reid, 2018). For example, increased meltability was
84 achieved by an increase in fat globule diameter (from 7.2 μm to 7.7 μm) in low salt low-moisture
85 Mozzarella cheese (1% salt, 4 °C storage; Ma, James, Balaban, Zhang, & Emanuelsson-Patterson,
86 2013) and increased meltability achieved in cheese with a more consistent homogeneous
87 microstructure with smaller protein strands and fewer serum pockets in direct-acidified no fat
88 Mozzarella cheese, where calcium was reduced through acetic acid addition to the cheese milk
89 (4 °C storage; McMahon, Paulson, & Oberg, 2005).

90 The functionality of low-moisture Mozzarella cheese is also known to change with time.
91 Meltability and stretchability increase in the initial 2-3 weeks following manufacture; a process
92 that is attributed to the absorption of free moisture by the protein matrix in the first two weeks
93 (McMahon, Fife, & Oberg, 1999). The meltability of Mozzarella cheese stored as a shredded
94 product has also been observed to increase over 40 d (Cortez et al., 2008). To date there are few
95 comparisons of the functionality of Mozzarella cheese that has been stored as a block or
96 shredded product. Storage of cheese as a shredded product has been reported to increase the
97 meltability of low-fat Mozzarella cheese (17% fat) in comparison to block cheese of the same
98 composition, with slight increases also noted in the stretchability, although the reasons for these
99 differences were not explored (Oberg et al., 1992).

100 While the existing literature indicates that there may be differences in the structure and
101 physicochemical properties of block and shredded Mozzarella cheese, further work is required to
102 directly compare Mozzarella cheese that has been shred using an industrially relevant process.
103 The present study aims to evaluate the microstructure, proteolysis and functionality of
104 industrially produced block and shredded low-moisture Mozzarella cheese after storage at 4 °C.

105 **2. Materials and methods**

106 **2.1. Cheese manufacture and composition**

107 Industrial *pasta filata* low-moisture Mozzarella cheese was collected from a single production
108 run at a cheese factory. Three 5 kg vacuum-packed blocks were obtained the day after
109 production to give three replicates. Each cheese block was divided into six equal pieces and
110 vacuum sealed and a new piece used for all analyses at each time period. Shredded cheese
111 samples were prepared by the same factory using cheese from the same production run. Blocks
112 were shredded 15 d and 8 weeks post manufacture (referred to as '15-d shred' and '8-wk shred'
113 respectively). The resultant cheese shreds in the 15-d shred and 8-wk shred ranged in length
114 from 2-10 cm with a diameter of approximately 0.5 cm. The first period was chosen after a short
115 storage time, common to many cheese processing factories, as this time period often coincides
116 with a low expressible serum (Kindstedt, 1995). The second period was chosen, as shredding
117 may also be conducted by the commercial customer months after the initial manufacture of the
118 cheese. Cheese blocks that were stored at 4 °C were transferred to an ambient temperature for
119 shredding. Cheese was shredded by a rotating blade and the shreds transported to a tumbling
120 drum system using a belt conveyor. In the drum, the cheese shreds were blended with 0.9-2.3%
121 w/w of a pre-blended anti-caking agent that contained 87% microcrystalline cellulose (MCC)
122 and 13% sorbic acid. The addition of anti-caking agent was manually adjusted by operators. The
123 coated shreds were sealed in bags (2 kg) with a modified atmosphere (70% N₂ and 30% CO₂).
124 Three 2 kg bags were collected for each period required for each treatment the day after
125 shredding (15-d shred n=9, 8-wk shred n=3). New bags of shredded cheese were used for
126 different time periods. All cheeses were stored at 4 °C for 8 weeks.

127 Fat content was determined using the Rose-Gottlieb method (IDF, 1996), protein content by the
128 Kjeldahl method (IDF, 1993), moisture content by oven drying (IDF, 1982) and salt

129 concentration by potentiometric titration with silver nitrate (IDF, 1988). The pH was measured
130 with a pH meter (Orion 720A, Orion Pacific Pty Ltd, Vic, Australia) by blending 20 g of grated
131 cheese with 10 mL of water (Australian Standard, 2010). The composition of block Mozzarella
132 and 15-d shred was measured after 15 d of storage at 4 °C. The composition of 8-wk shred
133 cheese was measured after 8 weeks of storage at 4 °C. The data are presented as the mean \pm the
134 standard deviation of three replicates for each of the three treatments (block, 15-d shred, 8-wk
135 shred).

136 **2.2. Water holding capacity**

137 Block cheeses were assessed for expressible serum (ES) content at 2, 9, 14, 17 and 21 d after
138 manufacture. A new cheese sample was used for each period, with the exception of the 9 d
139 sample, which was vacuum-sealed after analysis and used also for analysis on 14 d. 15-d shred
140 cheeses were assessed at 17 and 21 d post manufacture. Further testing was not conducted
141 beyond 21 d as ES was not expected to be measurable for older samples. A method similar to
142 that of Guinee *et al.* (2002) was applied, where freshly grated cheese (\sim 2 g) was prepared using
143 a hand-held grater, centrifuged using a micro-centrifuge (Model 5424, Eppendorf AG, Hamburg,
144 Germany) for 45 min at 20238 x *g* at 25 °C and the mass of serum, defined as the middle layer
145 extracted with a micropipette, measured. This weight of serum was expressed as a percentage of
146 total sample mass. The data presented are the mean \pm the standard deviation of three replicates.

147 **2.3. Microstructure**

148 Samples were prepared for microstructural analysis by confocal laser scanning microscopy
149 (CLSM; Leica Microsystems, Heidelberg, Germany) by cutting the cheese in either a parallel or
150 perpendicular orientation to observe the protein strands (Obergh, McManus, & McMahon, 1993),
151 using a method previously described (Ong, Dagastine, Kentish, & Gras, 2010). Microstructure
152 was assessed 2 days (block only), 9 days (block only), 3 weeks (block and 15-d shred) and 8

153 weeks (block, 15-d shred and 8-wk shred) post manufacture and images collected using a 63x
154 oil immersion objective. Three-dimensional (3D) image reconstructions of the CLSM
155 micrographs were conducted using Imaris software (Bitplane, CT, USA). Image analysis was
156 carried out on six images per period for each treatment (block cheese at day 2, day 9, week 3
157 and week 8, 15-d shred cheese at week 3 and week 8 and 8-wk shred cheese at week 8) as
158 described previously (Ong, Dagastine, Kentish, & Gras, 2012). The 2D images were initially
159 normalised to equalise the fluorescence intensity over the sample depth. A total of 40 2D images
160 512 x 512 pixels in size were taken at increasing sample depth 0.25 μm apart from an initial
161 depth of $\sim 10 \mu\text{m}$ below the sample surface. The surface area of the fat was determined from the
162 rendered surface of the stained fat. The data were averaged and are presented as the mean \pm the
163 standard deviation of six replicates for each of the three treatments.

164 **2.4. Proteolysis**

165 **2.4.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis** 166 **(SDS-PAGE)**

167 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess the
168 proteins and pattern of proteolytic degradation in cheese samples (Ong et al., 2010). SDS-PAGE
169 analysis was performed on samples collected after 2 days, 17 days, 4 weeks and 8 weeks of
170 storage at 4 $^{\circ}\text{C}$. A stock solution of each sample was prepared directly after each collection,
171 containing 25 mg of cheese, 1mL of Tris (10 mM) (Sigma-Aldrich, MO, USA)-EDTA (1 mM)
172 (Sigma-Aldrich) pH 8.0 buffer, 350 μL of 10% SDS (Sigma-Aldrich) and 50 μL of β -
173 mercaptoethanol (Merck, Darmstadt, Germany). The stock solution was frozen at -18 $^{\circ}\text{C}$ until all
174 samples were collected to enable samples to be analysed in the same gel and thawed
175 immediately prior to use. A molecular weight standard (SeeBlue Plus2, Invitrogen, Mt Waverly,
176 Australia) was loaded into the gel and protein bands were identified based on their estimated
177 molecular weight compared to the standard. Quantification of gel band intensities was carried

178 out using ImageJ (Research Services Branch, Natl. Inst. of Health, USA). Each lane was marked
179 and the band intensities plotted. The area of each peak was then calculated and the protein band
180 intensities presented as percentage of the band intensity obtained 2 d after manufacture.

181 **2.4.2. Reverse-phase high performance liquid chromatography** 182 **(RP-HPLC)**

183 Water-soluble peptides were analysed using reverse-phase high performance liquid
184 chromatography (RP-HPLC, Agilent 1100 Series, Agilent Technologies, CA, USA), with auto-
185 sampling and ultraviolet (UV) detection in order to further monitor proteolysis in the cheese
186 samples, by measuring total peak area determined by HPLC and to assess the relative changes in
187 hydrophobic and hydrophilic peptides, measured by total hydrophobic peak area and
188 hydrophilic peak area, as a function of storage. The water-soluble extract (WSE) was prepared
189 using an adaptation of a previously reported method (Ong, Henriksson, & Shah, 2006). Freshly
190 grated cheese (10 g) was blended at high speed (Polytron, Kinematica, Lucerne, Switzerland)
191 with 20 mL of water and incubated at 40 °C for 60 min, followed directly by centrifugation at
192 3200 x *g* at 4 °C for 20 min (model 5819 R, Eppendorf, North Ryde, Australia). The supernatant
193 was filtered (No 54 paper, Whatman, NSW, Australia) to give the WSE, which was further
194 centrifuged at 3200 x *g* at 4 °C and freeze-dried (Virtis Benchtop K, SP Scientific, PA, USA).

195 The WSE samples were analysed using RP-HPLC following a modification of a previously
196 reported method (Folkertsma, Fox, & McSweeney, 1996). Two solvents (A and B) were used for
197 gradient elution of proteins. Solvent A was 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich) in
198 water and solvent B was 0.1% TFA in acetonitrile (Merck). Freeze-dried WSE was diluted with
199 solvent A and filtered through a 0.45 µm polyvinylidene fluoride filter (Millipore, Vic, Australia).
200 The filtrate (20 µL) was analysed at room temperature by RP-HPLC with a 3.6 µm Aeris peptide
201 C18 column (250 x 4.6 mm) fitted with a guard column (2 x 4.6 mm; Phenomenex, NSW,
202 Australia). The flow rate was 0.75 mL/min with 100% solvent A for 5 min, followed by a

203 gradient from 0% to 80% solvent B for 88 min, elution at 80% solvent B for 6 min, followed by a
204 gradient from 80% to 90% solvent B for 10 min, 90% solvent B for 3 min and a gradient from
205 90% to 0% solvent B for 2 min to return to column to solvent A, prior to cleaning and the next
206 sample run. The detection wavelength was 280 nm. The location of hydrophilic and
207 hydrophobic peptides were defined as peaks that eluted from 0 – 35 min and 35 – 80 min,
208 respectively (de Llano, Polo, & Ramos, 1995). The areas of the peaks were calculated for both
209 the hydrophilic and hydrophobic peptides to enable relative comparisons as a function of
210 storage time and are presented as the means \pm the standard deviation of three replicate
211 measurements.

212 **2.5. Casein zymography**

213 The effect of the storage format and storage conditions (modified atmosphere packaging and
214 anti-caking agent) on proteolysis within the cheese was also assessed by zymography. Cheese
215 was stored as a vacuum-packed block for 15 d after manufacture. Cheese samples shredded at
216 15-d were either blended with anti-caking agent and stored in a modified atmosphere stored
217 without anti-caking agent in the absence of a modified atmosphere. Block samples were stored
218 for the remaining storage period in vacuum packaging. Samples were stored at 4 °C and
219 analysed 8 weeks after manufacture for enzyme activity.

220 Samples were prepared for casein zymography by mixing 5 μ L of WSE (prepared as described in
221 Section 2.4) with 5 μ L of Novex Tris-Glycine SDS sample buffer (Life Technologies, CA, USA).
222 Samples were separated on a 12% acrylamide gel containing 0.1%, w/v casein (Sigma-Aldrich)
223 to provide a substrate for enzyme hydrolysis; this protein leads to staining of the entire gel in
224 later steps, except for those regions where proteolysis of casein has occurred. A constant voltage
225 of 90 V was applied. The gel was developed for 30 min in a Novex developing buffer (Life
226 Technologies), then incubated with a Novex Zymogram renaturing buffer (Life Technologies)
227 overnight at 37 °C and finally stained with Coomassie Blue (Bio-rad, NSW, Australia), as

228 described previously (Ong et al., 2010). Gels were visualised as described in Section 2.4.1. Any
229 areas of clearance arising from enzyme activity were selected (with a box around this area) and
230 the percentage intensity measured was expressed as a percentage of background intensity to
231 provide an estimate of enzyme activity. Image processing was conducted over the entire area of
232 the gel to improve the appearance of the features (20% increase in brightness, 40% increase in
233 contrast). The data presented are representative of two replicates.

234 **2.6. Functionality tests**

235 The meltability of all cheese samples was measured by the modified Schreiber test (Kosikowski
236 & Mistry, 1997) after 2, 3, 4 and 8 weeks of 4 °C storage. A cylinder of cheese 10 mm thick and
237 36 mm in diameter was heated at 130 °C for 10 minutes in a conventional oven (S.E.M. Pty. Ltd.,
238 Magill, Australia). For block samples the cylinder was cut from the block. For 15-d and 8-wk
239 shredded cheese samples, the shreds were manually pressed into a disc of cheese of the same
240 dimensions and a similar weight to the block cheese to enable comparison. After the cheese had
241 cooled to room temperature, the area of the melted cheese was measured by averaging the
242 radius taken at six different points around the cheese. The data presented are the mean \pm the
243 standard deviation of three replicates for each of the three treatments.

244 An additional melt test was used for block cheese samples only after 1, 2, 3, 4 and 8 weeks of 4
245 °C storage. Samples of 36 mm diameter and ~4 mm thickness were prepared and the meltability
246 assessed using a controlled stress rheometer (AR-G2, TA Instruments, Delaware, USA) (Ma et al.,
247 2013). A temperature sweep test was conducted from 10 °C to 90 °C at a constant 0.05% strain
248 and 0.8 Hz in the linear viscoelastic region. The crossover between the elastic modulus (G') and
249 the viscous modulus (G''), defined as the transition temperature, was determined from this test.
250 The data presented are the mean \pm the standard deviation of three replicates for each of the
251 three treatments.

252 The stretchability of block and shredded Mozzarella cheese was assessed using an Instron 5944
253 (Instron Corp., Norwood, USA) fitted with 2 kN load cell and rubber-coated grips. A mass of 25 g
254 of shredded cheese was melted onto a cracker (Salada; dimensions 9 cm x 10 cm; Arnotts
255 Biscuits Ltd, NSW, Australia) at 240 °C (Sunbeam BT5350 Toaster Oven, NSW, Australia). Block
256 cheese samples were shredded prior to the test using a household food processor (Model
257 FP14MRA; Cuisinart, Ontario, Canada) fitted with a shredding blade with 4 mm openings. The
258 cheese shreds produced were 3-7 cm in length and 0.2-0.5 cm in diameter. The cheese cracker
259 was then carefully broken into two halves along the perforated line and rapidly transferred to
260 the machine and secured within the grips. The instrument then pulled apart the halves to a total
261 distance of 400 mm and the force (N) required to move the cheese and final length of the melted
262 cheese mass were measured. The maximum force recorded during the stretch test was
263 determined to be the 'melt strength.' The stretch quality was assessed as the force required to
264 stretch the sample a distance of 50 mm or 300 mm. The stretch length was defined as when all
265 the cheese strands broke or the end of the test (400 mm). The data presented are the mean \pm the
266 standard deviation of three replicates for each of the three treatments.

267 The pizza bake test was conducted using a method adapted from Soodam (2015) after 1, 3, 4
268 and 8 weeks of storage at 4 °C. One frozen-thawed pizza base (23 cm in diameter; (McCains, Vic,
269 Australia) was evenly coated with 90 g of tomato paste (Leggos, Vic, Australia). The block cheese
270 was shredded on the same day using a food processor using the same setting for the
271 stretchability test (Section 1.2) and 100 g of the shredded cheese was evenly dispersed over half
272 of the pizza. The pizza was baked at 240 °C in a fan-forced oven (Model E33T5, Turbofan, Moffat,
273 Vic, Australia) for a set period of 8 minutes for both treatments. The pizza was rotated 180
274 degrees to ensure even cooking after the first four minutes. Assessments were performed
275 empirically by a trained non-blinded judge as an indicator of quality control processes
276 performed in industry; the baked pizza was visually evaluated for melt (the extent of integration
277 of the separate shreds), free oil release (the volume of fat released on the surface), browning

278 (the colour of the cheese) and blistering (the elevated areas of melted cheese). A 4-pronged fork
279 was used to evaluate skinning (the extent to which cheese pulls across the surface) and stretch
280 (the distance the cheese strands could be pulled vertically from the pizza base). Each image of a
281 pizza contains two replicates where one replicate covers half a pizza only.

282 **3. Results and Discussion**

283 **3.1. Cheese composition**

284 The composition of the cheese studied here (Table 1), was typical of *pasta filata* low-moisture
285 Mozzarella (Kindstedt, Carić, & Milanović, 2004). Cheese was shredded and treated with a small
286 mass of pre-blended microcrystalline cellulose (MCC) (0.9-2.3% w/w) prior to packing in a
287 modified atmosphere. The composition of the shredded product remained within typical
288 manufacturing targets, although some variability in fat was observed, potentially due to the loss
289 of fat during shredding and handling. After 8 weeks the pH of both block and samples that had
290 been shredded after 15 d and 8 wks was ~5.5-5.6, similar to the pH measured for a block of
291 Mozzarella, after 40 days of storage at 4°C in a prior study (Smith et al., 2017).

292 **3.2. Water holding capacity**

293 The expressible serum within the cheese was measured as an indication of free moisture prior
294 to and at the point of shredding. At day two, a high level of serum ($20.6 \pm 1.2\%$) could be
295 expressed (Fig. 1), indicating that the serum was not well bound to the protein network. The
296 reported values of expressible serum in the initial days following manufacture of low moisture
297 Mozzarella vary from 10% (Kuo, Gunasekaran, Johnson, & Chen, 2001) up to 30-40% (Guo &
298 Kindstedt, 1995; O'Reilly et al., 2002), reflecting variations in cheesemaking parameters and the
299 conditions used to express serum.

300 In this study, the expressible serum declined in the first two weeks after manufacture (Fig. 1),
301 potentially as the protein matrix became more hydrated, consistent with the literature
302 (McMahon et al., 1999). Only 0.2-1.6% of serum was quantified in block samples after this
303 period. The shredding times of 15 d and 8 weeks were selected to coincide with the expected
304 drop in expressible serum after the protein network has absorbed available water, as free
305 moisture causes the cheese to stick to the blade during shredding (Kindstedt, 1995). Industrial
306 shredding also typically occurs after the expressible serum has been absorbed by the protein
307 network and the shredded samples tested at days 17 and 21 gave no measurable expressed
308 serum.

309 **3.3. Microstructure**

310 The microstructure of the block Mozzarella samples after manufacture consisted of continuous
311 aligned protein phase with an entrapped elongated fat phase (Fig. 2). Some coalesced fat was
312 also present in the serum phase. 3D confocal laser scanning microscopy (CLSM) images were
313 collected and reconstructed and three views of the microstructure are presented in Fig. 2; the X-
314 Y image (the main image), the X-Z image (the narrow image at the bottom) and the Z-Y image
315 (the narrow image on the right). These three different perspectives confirm the microstructure
316 observed in the direction of the Y and Z axes is consistent with the main X-Y image. The fat
317 droplets ranged in size from $\sim 20\ \mu\text{m}$, for the larger coalesced droplets, to fat less than $1\ \mu\text{m}$ in
318 diameter and clusters of these smaller fat droplets had a diameter of $\sim 5\ \mu\text{m}$.

319 The presence of serum soon after manufacture is consistent with the observations of
320 industrially produced Mozzarella cheese by CLSM and scanning electron microscopy (SEM;
321 McMahon et al., 1999; Smith et al., 2017) and earlier SEM analysis of the microstructure of pilot
322 scale Mozzarella cheese (Kiely et al., 1993), together with the high level of expressible serum
323 measured here for these samples (Fig. 1). The size distribution of fat globules present is also

324 similar to the range of fat globule sizes found in a model Mozzarella that had been subject to
325 work in the *pasta filata* process (Sharma, Munro, Gillies, Wiles, & Dessev, 2017).

326 The presence of serum is also an indication of the high shear applied during manufacture.
327 Sharma and colleagues found that as shear increased, serum was expelled by the protein
328 network in Mozzarella (2016). Higher shear also led to a loss of anisotropy in the protein
329 network (Sharma et al., 2017). Laminar mixing of the cheese, initially at low shear, led to the
330 formation of fat channels and alignment of protein structure but this was followed by the
331 disappearance of alignment at higher shear, suggesting that these industrial samples, shown in
332 Fig. 2, were similarly subject to a high level of shear.

333 The free moisture in the serum appeared to be absorbed with storage, as this was less visible in
334 block samples at later time periods (Fig. 2), consistent with the reduction of expressible serum
335 (Fig. 1) and observations of absorption in other studies of Mozzarella during storage (Kuo et al.,
336 2001; McMahon et al., 1999), including an increase in water associated with protein detected by
337 ^1H NMR (Smith et al., 2017).

338 Channels containing small fat droplets were visible from day 9, where the fat appeared to
339 cluster closely. The formation of these channels suggests the rearrangement of the protein
340 phase into a more striated structure, potentially as the network recovers from the shear
341 experienced during production and absorbs free moisture. A slow rearrangement of
342 microstructure is common for many biopolymer networks exposed to shear (Frith, 2010).
343 Extruded foods are also known for their non-equilibrium nature, where the protein network
344 may rearrange with time (Tolstoguzov, 2002).

345 The alignment of the protein phase was still visible at week 8 and other studies have observed
346 parallel alignment of the protein phase and fat for Mozzarella cheese up to 5 weeks after
347 manufacture (Guinee et al., 2002; Kiely et al., 1993). The fat also appeared relatively stable in
348 these samples. One prior study observed evidence for the potential coalescence of fat after 50 d

349 of refrigerated storage (Kiely et al., 1993). While some degree of coalescence was observed in
350 the current study, the fat droplets appear largely clustered within channels, perhaps explained
351 by the differences in imaging techniques (confocal here compared to SEM of defatted samples in
352 the prior study).

353 The shredded samples had a microstructure that differed to the microstructure of the block
354 samples after 8 weeks of storage (Fig. 2A), this included both the 15-d shred samples that were
355 aged to 8 weeks and the 8-wk shred samples that were shredded at 8 weeks. The fat droplets in
356 these samples were more dispersed and did not appear to be arranged in channels. At the
357 earliest period of 3 weeks after manufacture, the microstructure of the 15-d shred samples and
358 block samples were similar, suggesting the handling and shear of shredding process did not
359 alter the structure of the cheese at this early stage and that the effects observed at 8 weeks were
360 a combination of shredding and aging of the samples.

361 Differences in the microstructure of the fat in the shredded samples can be further examined by
362 image analysis, where the distribution of fat can be quantified using the total surface area of all
363 fat clusters, or droplets, in a 3D volume of cheese. In cheese where the fat droplets cluster, a low
364 total surface area of fat is detected per unit volume when image analysis is performed. In
365 contrast, dispersed fat droplets result in a higher total surface area of fat per unit volume of
366 cheese. Eight weeks after manufacture, the total surface area of the fat was higher per unit
367 volume of cheese in the shredded samples compared to the block samples (Fig. 2B), consistent
368 with the visual observation of fat appearing more dispersed in these samples.

369 The analysis of Mozzarella by CSLM shows changes in the microstructure of the block occurring
370 as a function of sample storage at 4°C, including the disappearance of serum and clustering of fat
371 in channels that may occur due to the absorption of water and slow rearrangement of cheese
372 protein phase after shear. Differences are also observed in the microstructure at 8 weeks
373 between block and shredded product, as a result of shredding and aging Mozzarella cheese. The

374 fat is more dispersed, with a higher total surface area of fat per unit volume of cheese in these
375 shredded samples. The mechanism behind the changes observed in the shredded product
376 microstructure are not clear from the images alone. The relaxation of the protein phase
377 observed in the block Mozzarella (Fig. 2) may be altered by shredding. Changes in the
378 biochemistry of the cheese as a result of shredding, increased handling, incubation with MCC,
379 modified atmosphere packing and also aging may also be responsible for these changes. For
380 example, the hygroscopic MCC within the anti-caking agent could shift the water distribution in
381 the samples, leading to a structural alteration. Increased proteolysis in the shredded samples
382 could also result in a rearrangement of the protein network in these samples.

383 **3.4. Proteolysis**

384 Proteolysis was monitored by two complementary methods, SDS-PAGE and RP-HPLC, to assess
385 the effect of shredding on the extent of protein breakdown either from the proteolytic action of
386 starter culture or non-starter flora and plasmin present in the Mozzarella cheese. These
387 methods involve analysis of the α - and β -caseins within the cheese (SDS-PAGE) and the
388 proteolytic breakdown products within the WSE (RP-HPLC). The intensity of the bands
389 corresponding to α -caseins and β -casein were monitored by SDS-PAGE as a function of storage
390 time (Fig. 3). Freshly manufactured cheese assessed at 2 days after manufacture contained a
391 wide range of proteins, as expected (Upadhyay, McSweeney, Magboul, & Fox, 2004). A high
392 proportion of this protein was α -casein (45-55% of total protein) or β -casein (25-35% of total
393 protein), other proteins present included, for example, α -lactoglobulin and γ^2 and γ^3 casein.

394 After 8 weeks of storage, a much lower proportion of the α -caseins present in shredded cheese
395 remained ($61 \pm 2\%$) compared to cheese stored for the same period as a block product ($91 \pm$
396 6%) or cheese stored as a block and then shredded at 8 weeks ($84 \pm 4\%$), although this analysis
397 of SDS-PAGE band intensity is semi-quantitative (Fig. 3b). The β -casein fraction underwent less

398 hydrolytic breakdown during storage and the amount of β -casein remaining after 8 weeks was
399 similar for all three treatments (Fig. 3c).

400 The proportion of α -caseins remaining here after 8 weeks of storage (61-91%) is consistent
401 with the proportion of α -caseins remaining for block Mozzarella cheese (60-76%) produced
402 with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* and stored for 6-8 weeks under
403 conditions similar to those used here (Imm et al., 2003; Tunick et al., 1993). Proteolysis can
404 differ between studies, however, because of different starter cultures, non-starter flora,
405 coagulants or the presence of plasmin, which can vary between manufacturers. The lower level
406 of α -caseins remaining compared to β -casein observed here is also expected for the lactic acid
407 starter culture used (Farkye, 2014).

408 Two related measures of proteolysis, total peak area and the hydrophilic peak area of peptides
409 in the water soluble extracts, were higher after 8 weeks of storage for shredded cheese ($5.6 \times 10^3 \pm 2.1 \times 10^3$ mAu.min and $5.4 \times 10^3 \pm 2.0 \times 10^3$ mAu.min) compared to cheese stored in a block
410 ($1.0 \times 10^3 \pm 0.25 \times 10^3$ mAu.min and $0.87 \times 10^3 \pm 0.36 \times 10^3$ mAu.min). Cheese that had been
411 stored and then shredded at 8 weeks similarly had a higher total peak area ($8.2 \times 10^3 \pm 1.7 \times 10^3$
412 mAu.min) and hydrophilic peak area ($7.7 \times 10^3 \pm 0.96 \times 10^3$ mAu.min). In contrast, the
413 hydrophobic peak area was similar for all treatments after 8 weeks of storage.

415 The low levels of proteolysis in block samples, measured here at the 8th week, is consistent with
416 prior studies of block cheese using *S. thermophilus* as a starter culture, as employed in the
417 present study, where water soluble peptides were produced after 8 weeks of storage (Oommen,
418 McMahon, Oberg, Broadbent, & Strickland, 2002).

419 There is scanty literature on the rates of proteolysis in shredded cheese. One study of
420 Mozzarella cheese with a composition comparable to the present study reported similar levels
421 of proteolysis for block or shredded cheese that had both been vacuum-packed; storage was at 4
422 °C for 7 weeks (Cortez et al., 2008), however, these samples were shredded earlier than here (2

423 d compared to 2 weeks or 8 weeks) and employed a different method to assess proteolysis.
424 Another difference between these two studies was the use of both a modified atmosphere (70%
425 N₂ and 30% CO₂) and the addition of anti-caking agent (0.9-2.3% w/w microcrystalline cellulose
426 (MCC) containing 13% sorbic acid) to shredded samples in the current study, to reflect the
427 conditions often used commercially to preserve shredded cheese.

428 The higher level of proteolysis measured in the shredded samples (Fig. 3) suggests this
429 biochemical change may lead to the rearrangement of the protein network and changes in fat
430 distribution observed in the shredded cheese (Fig. 2). Proteolysis is known to soften the cheese
431 and to alter protein networks in Mozzarella (Imm et al., 2003) and can increase the capacity of
432 the protein network to bind water (Ardö, McSweeney, Magboul, Upadhyay, & Fox, 2017).
433 Changes in the structure of the network and casein-water interactions (Lamichhane, Kelly, &
434 Sheehan, 2018), where substantial, can also lead to a change in functional properties.

435 **3.5. Casein zymography**

436 Casein zymography was performed in order to detect proteases present in block and shredded
437 cheese samples. The effect of treating shredded samples with MCC and storing these samples in
438 MAP was also assessed.

439 Two proteases less than 250 kDa in molecular weight were observed in the water-soluble
440 extract of the block cheese and 15-d cheese shred samples. When assessed by casein
441 zymography at 8 weeks of storage, these were visible as a lighter band of clearance in contrast
442 to the darker background (Fig. 5a). Potential proteolytic enzymes can be separated and visually
443 identified using zymography, as the enzymes clear casein in their immediate vicinity within the
444 zymogram gel. The two bands occurred at ~43 kDa and ~51 kDa. One possible identity for the
445 ~43 kDa band is the intracellular enzyme aminopeptidase PepS that has been characterised
446 from the starter culture *S. thermophilus* used here (Savijoki, Ingmer, & Varmanen, 2006). The
447 ~51 kDa band could also correspond to an intracellular enzyme of *S. thermophilus* (Husson-Kao

448 et al., 2000; Lortal & Chapot-Chartier, 2005). Intracellular enzymes, including proteases, are
449 typically released when such bacteria undergo autolysis (Lortal & Chapot-Chartier, 2005),
450 although extracellular proteases can also contribute to proteolysis.

451 Shredded samples had a higher level of proteolytic activity and band clearance than the block
452 samples, as shown in Figure 5b. Approximately 7% of the casein present in the zymograph gel
453 was cleared by both the ~51 kDa and the ~43 kDa enzymes in shredded samples, shredded at
454 either 15-d or 8 wk, compared to ~3% of casein hydrolysis by the two enzymes in block
455 samples. This higher enzymatic activity is consistent with the generally higher level of
456 proteolysis observed in shredded samples after 8 weeks by SDS-PAGE (Fig. 3) and HPLC (Fig. 4)
457 and the two enzymes observed by zymography may be responsible for the differences in
458 microstructure observed within the two types of shredded samples after 8 weeks (Fig. 2).

459 The clearance of casein in the zymograph gel was similar for shredded samples regardless of
460 whether they were stored with anti-caking agent containing MCC and in a modified atmosphere
461 or whether they received neither treatment (Fig. 5a). This observation indicates these
462 treatments, conducted as part of the shredding and storage process, did not significantly alter
463 the type of proteolytic enzymes present or the level of enzyme activity. This includes any
464 potentially differences in water movement, as a result of addition of hygroscopic MCC, which
465 appear not to have altered the enzyme activity in the cheese. Several studies have probed the
466 effect of MAP on the growth of bacteria and level of proteolysis. The growth of lactic acid
467 bacteria is not typically affected by the presence of a modified atmosphere, consistent with the
468 observations here (Eliot et al., 1998). There are, however, examples where MAP has been shown
469 to either reduce or increase proteolysis (Khoshgozaran, Azizi, & Bagheripoor-Fallah, 2012). A
470 future study could therefore probe the individual effect of MCC addition and MAP, to provide
471 further insight into the mechanisms of proteolysis in shredded samples observed here.

472 Some enzyme activity was detected at the top of the denaturing resolving gel from proteases
473 with a molecular weight >250 KDa. These large enzymes may be within disulfide bonded
474 protein complexes, as the conditions applied here were non-reducing. These complexes were
475 not the focus of this study, as proteolytic enzymes from LAB are reported to be less than 60 kDa
476 in size (Lortal & Chapot-Chartier, 2005).

477 **3.6. Functionality**

478 The functional properties of the block and shred samples including: meltability, stretchability
479 and performance in a pizza bake test were assessed at specific times over the 8-week storage
480 period (2, 3, 4 and 8 weeks). These tests, which include measures typically performed within
481 industry, were performed to provide an initial assessment of cheese quality and to determine if
482 shredding affected these functional properties.

483 Block and shredded cheese samples had a similar meltability, as measured by the modified
484 Schreiber test, with a relatively high level of variability in melted cheese area recorded for each
485 sample (Fig. 6a). The Schreiber test is often used in the literature; some studies report an
486 increase in meltability of Mozzarella cheese in the first three weeks following manufacture
487 (McMahon et al., 1999) and several other studies report stable meltability in the first one to two
488 weeks following manufacture (Andreatta et al., 2009; Rudan & Barbano, 1998). The variability
489 measured in the Schreiber test is not often reported, although one study found cheese with a
490 similar composition to the present study to have a relatively constant meltability, within the
491 levels of variation, over a storage period of 40 days at 4 °C (Cortez et al., 2008), highlighting the
492 potential importance of reporting variability in this test.

493 A further measure of meltability, the transition temperature, was used to assess the
494 performance of block samples as a function of storage time (Fig. 6b). The temperature at which
495 the block cheese transitioned from solid to liquid on heating in a rheometer decreased as a
496 function of storage time, indicating changes in the cheese properties during refrigerated storage.

497 While this rheological test is typically performed on a block of cheese and was not applied to the
498 shredded product, it does appear more sensitive than the Schreiber test and potentially a useful
499 measure of changes in block Mozzarella on storage. This test has also been shown to be sensitive
500 to other processing changes including the draining pH or the concentration of calcium (Ma,
501 James, Zhang, & Emanuelsson-Patterson, 2011).

502 The block and shredded samples stretched to a similar extent as assessed by melt strength,
503 stretch quality and stretch length (Fig. 7). The block samples also became easier to stretch over
504 the first two weeks following manufacture, as shown by a decrease in stretch quality, which
505 measured the force required to stretch samples to 300 mm. This latter observation is consistent
506 with previous reports that the stretchability of Mozzarella increases in the first two to three
507 weeks post manufacture (Guinee, 2002; Kuo & Gunasekaran, 2003). There are many variables
508 that affect the degree of stretchability, however, such as stretching speed, the amount of cheese
509 used for a stretch test, test temperature and oven type, making it difficult to directly compare
510 results from different methods (Lucey, Johnson, & Horne, 2003).

511 The melt, free oil release, browning, blistering, skinning and stretch properties of the block and
512 shredded cheese samples were similar when assessed in a pizza bake test (Fig. 8).
513 Improvements in the melt, browning, blistering and skinning were observed for the block
514 samples after three weeks of storage at 4 °C, likely arising from the changes in expressible
515 serum (Fig. 1) and rearrangement of the cheese microstructure (Fig. 2) over this period. The
516 pizza bake results were also generally consistent with prior reports, although the oven type and
517 baking conditions have a great impact on the properties of the final baked pizza (Chen, Wolle, &
518 Sommer, 2009). The browning and blistering displayed here were to a moderate extent reduced
519 compared to in some studies that reported baked pizza images (Ma et al., 2013; Zisu & Shah,
520 2005), as a turbo fan oven was used rather than an impinger-style pizza oven.

521 Block and shredded samples displayed similar functionality, including meltability, melt strength,
522 stretch quality and stretch length and pizza bake properties (Fig. 6-8) despite the differences in
523 microstructure (Fig. 2), proteolysis (Fig. 3 and 4) and enzyme activity (Fig. 5) observed in these
524 samples after 8 weeks of storage. This similarity may be due to the relatively lower sensitivity
525 and high variability of these functionality tests. These tests provide an initial indication,
526 however, that product quality may not be adversely affected by shredding at this time period. .
527 Further analysis of the microstructure, proteolysis and functionality of the shredded product
528 over longer periods of storage, across the product shelf-life, would be informative, however,
529 differences may become apparent at later time points.

530 Another notable observation, across the functionality tests performed, is that the cheese
531 product shredded at 15 d or 8 weeks had very similar functionality for all measures (Fig. 6-8).
532 This observation suggests there is flexibility in the timing of the shredding operation; this may
533 also allow product to be released for sale earlier.

534 Given the changes observed in shredded product in this study (Fig. 2-5), there is scope to
535 broaden our understanding of the impact of shredding further, by studying of a wider range of
536 shredding times. The samples examined here are also, by necessity, a small volume of the batch
537 of cheese manufactured and some heterogeneity is also expected at an industrial scale. The
538 further study of a greater number of shredded cheese samples from multiple manufacturing
539 runs would potentially enable the findings of this study to be more broadly applied at industrial
540 scale.

541

542 **4. Conclusions**

543 The microstructure, proteolysis and functionality of low-moisture Mozzarella cheese from the
544 same production batch stored at 4°C as a vacuum-packed block product or shredded at 15 d and

545 8 weeks product, coated in MCC and stored in MAP were compared. Shredding was found to
546 induce microstructural changes, as the fat appeared more dispersed compared to the channels
547 of fat clustered within the block cheese. Proteolysis increased in the shredded samples and a
548 higher level of activity was detected for two bacterial proteases, which may lead to the softening
549 and rearrangement of the protein network within the shredded cheese. Despite these changes,
550 the functionality of the cheese was preserved, indicating that shredding did not largely alter the
551 properties of the final product. Over this sampling period, cheese shredded at 15 d or 8 weeks
552 also had very similar functionality, suggesting there is flexibility in the timing of the shredding
553 operation.

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Table 1: The composition of Mozzarella cheese samples (mean \pm the standard deviation of three replicates)

Sample	Fat (g/100 g)	Protein (g/100 g)	Moisture (g/ 100 g)	Salt (g/100 g)	pH
Block	22.4 \pm 0.6	23.5 \pm 0.1	48.7 \pm 0.2	1.8 \pm 0.2	5.5 \pm 0.04
15-d shred	20.7 \pm 0.7	23.7 \pm 0.3	48.4 \pm 0.1	1.5 \pm 0.1	5.5 \pm 0.04
8-wk shred	20.7 \pm 0.4	23.9 \pm 0.3	47.9 \pm 0.9	1.6 \pm 0.1	5.6 \pm 0.08

Figure captions

Figure 1: Expressible serum from stored block Mozzarella cheese (mean \pm the standard deviation of three replicates).

Figure 2: **(A)** 3D confocal micrographs of the microstructure of block (**a, b, c, e**), 15-d shred (**d, f**) and 8-wk shred (**g**) Mozzarella cheese at day 2 (**a**), day 9 (**b**), week 3 (**c, d**) or week 8 (**e to g**). Samples were sectioned and imaged parallel to the protein strands. Each image is presented in three views; the *X-Y* image (main image), the *X-Z* image (narrow bottom image) and *Z-Y* image (narrow right image). Fat in these samples stained with Nile red appears red and protein stained with fast green FCF appears green. The scale bars are 20 μm in length. **(B)** Total surface area of fat within in the 3D confocal micrographs for the block (\blacklozenge), 15-d shred (\square) and 8-wk shred (\blacktriangle) samples. Each 3D image used for analysis is a stack of 40 adjacent layers with a total depth of 10 μm . The gap between layers was a constant 0.25 μm . Error bars represent the standard deviation of two independent measurements from each of the three replicate samples..

Figure 3: **(a)** SDS-Page analysis of block (B), 15-d shred (15-d) or 8-wk shred (8-wk) Mozzarella cheese analysed after 2 d, 17 d, 4 weeks and 8 weeks of storage at 4 °C. Protein bands were identified according to their molecular weights, with reference to the molecular weight standard (Std on gel) and by comparison to the α_s , β , and κ casein standards (α_s , β , and κ CN on gel).. The corresponding changes in α - and β -caseins with storage (**b, c**) were calculated from the intensity of band staining in the SDS-PAGE profile for block (\blacklozenge), 15-d shred (\square) and 8-wk shred (\blacktriangle) cheese samples and are expressed as a percentage of the remaining casein in the cheese at day 2. Error bars are \pm the standard deviation of two samples, except for week eight where four samples were analysed.

Figure 4: Changes to the HPLC peak area corresponding to: **(a)** the total peak area, **(b)** hydrophobic peptides and **(c)** hydrophilic peptides for the water soluble extract prepared from

block (◆), 15-d shred (□) and 8-wk shred (▲) cheese as a function of storage time. Data points are the mean ± the standard deviation of three replicates.

Figure 5: Casein zymography profiles of Mozzarella cheese at the eighth week after manufacture for cheese stored as a block or shredded product. (a) Bands appear at ~51 kDa and at ~43 kDa due to proteolysis and clearance of the local area of the gel near enzymes. MW refers to the molecular weight marker. Samples from left to right were the block cheese, the cheese shredded at 15 days and the same cheese shredded at 15 days but not treated with anti-caking agent nor stored in modified atmosphere packaging. The band clearance (%) in the zymograph gel for each of the two areas at ~51 kDa and at ~43 kDa was calculated quantitatively by measuring the intensity of the cleared areas relative to the background intensity (b) for the block cheese (◆), the cheese shredded at 15-d (□) and the cheese shredded at 15-d with no anti-caking agent or modified atmosphere packaging (●).

Figure 6: Meltability of Mozzarella samples as measured by (a) the modified Schreiber method for block (◆), 15-d shred (□) and 8-wk shred (▲) and (b) the transition temperature determined by rheological analysis (block samples only). The data presented are the mean ± the standard deviation of three replicates, except for the transition temperature at 1 week where only three samples displayed a transition temperature.

Figure 7: Stretchability of Mozzarella samples: a) Melt strength, b) Stretch quality at 50 mm and 300 mm and c) Stretch length for ◆) block cheese, □) 15-d shred cheese, and ▲) 8-wk shred cheese. Solid lines in the graph (b) represent the stretch quality at 50 mm and dotted lines the stretch quality at 300 mm. The data presented are the mean ± the standard deviation of three replicates.

Figure 8: Visual appearance of cheese used as a pizza topping upon baking at 240 °C for 8 minutes in a turbo fan oven for block (a, b, d, f), 15-d shred (c, e, g) and 8-wk shred (h)

Mozzarella cheese after one (**a**), three (**b, c**), four (**d, e**) and eight (**f to h**) weeks of storage post manufacture. Each pizza half is covered with one replicate sample.

Figure 1

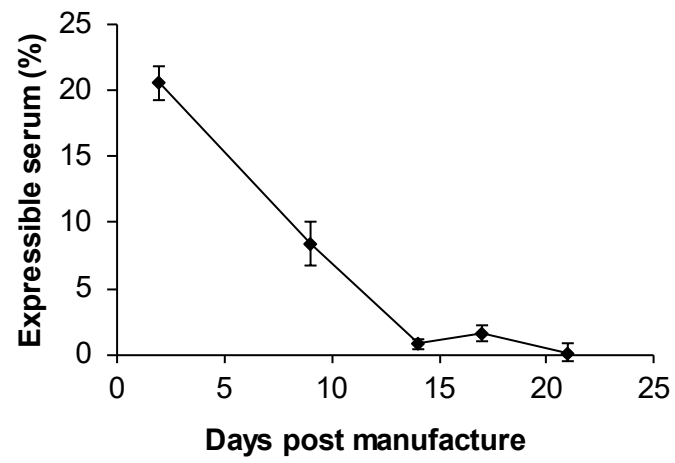


Figure 2

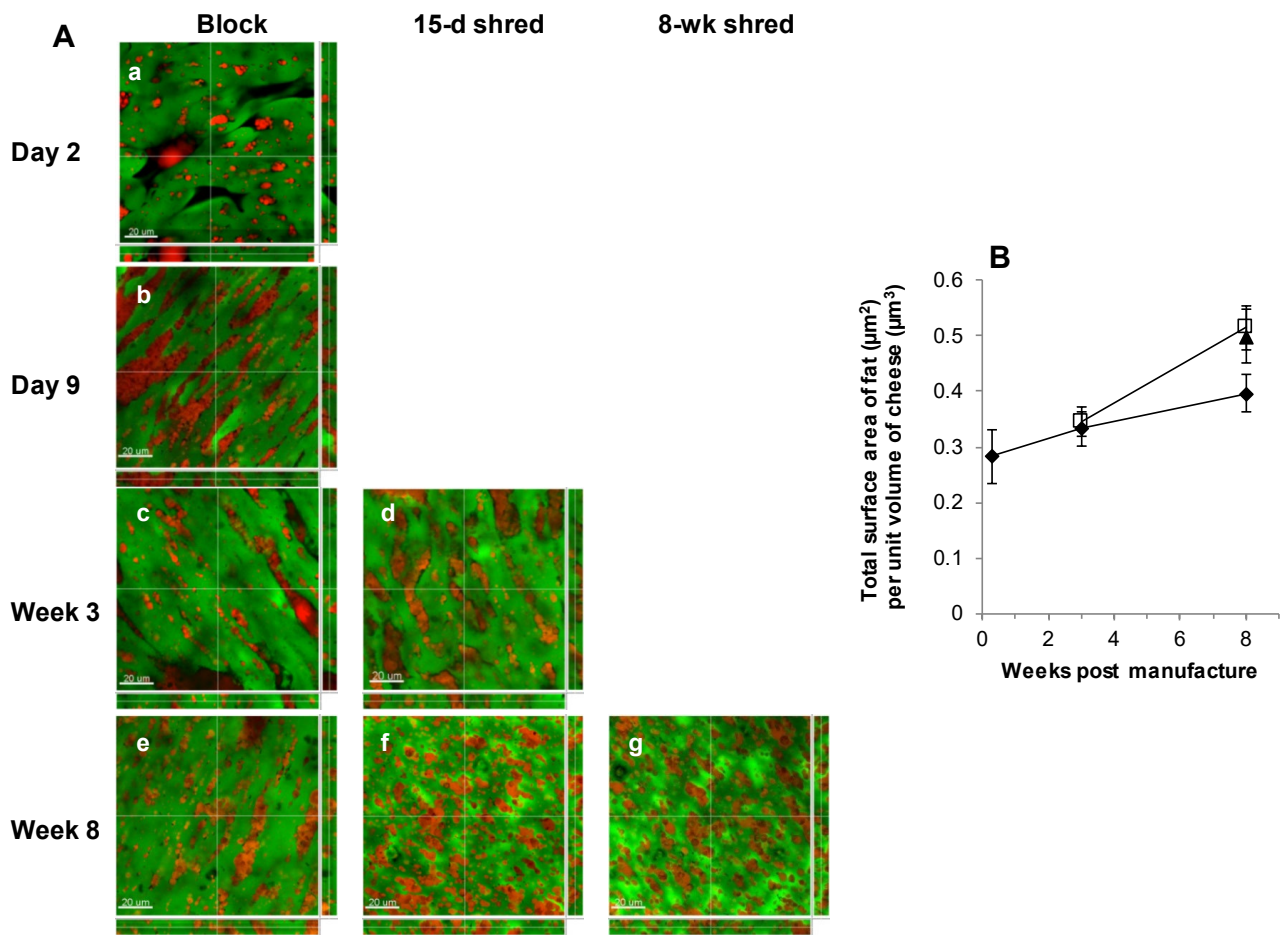


Figure 3

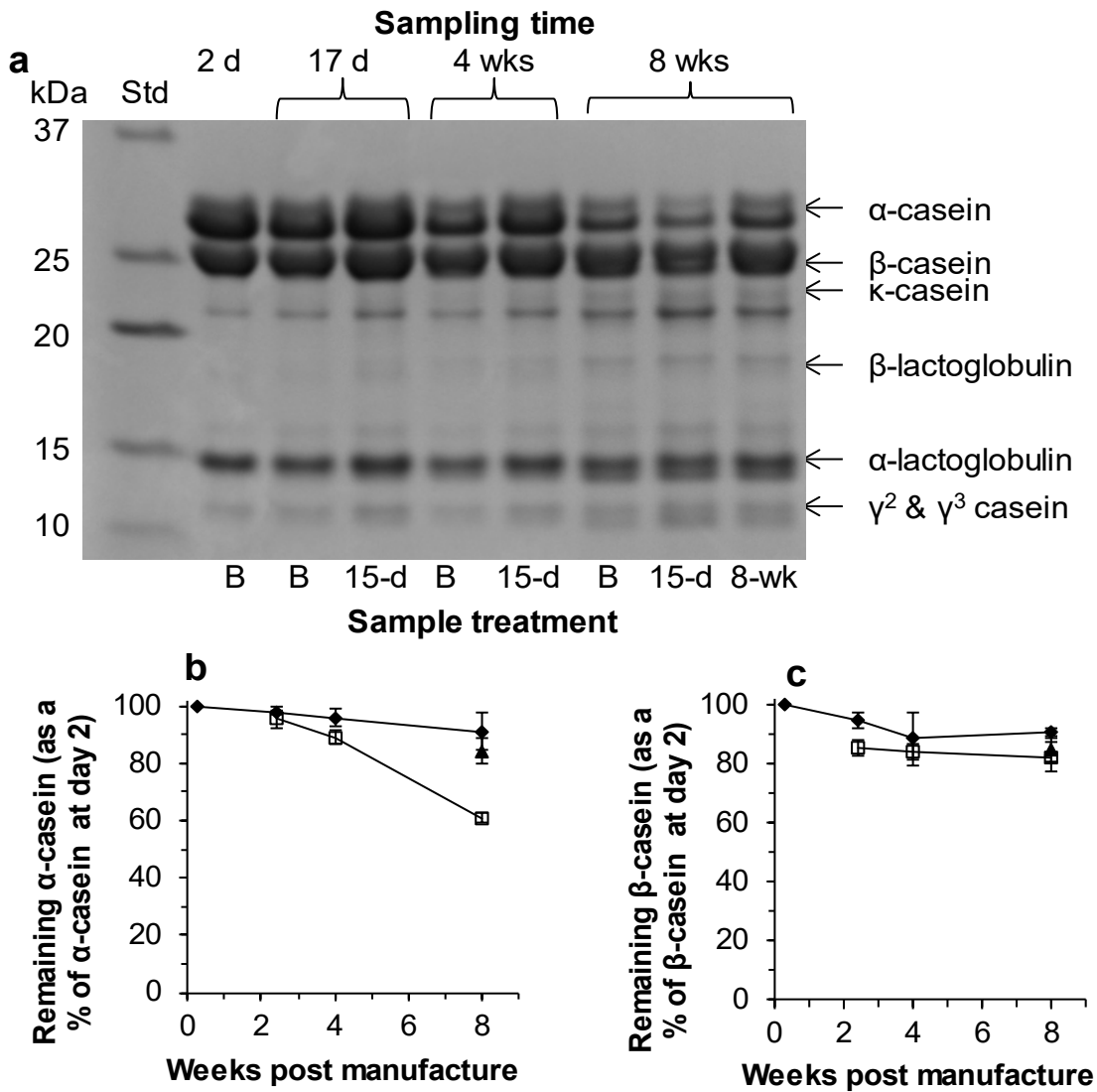


Figure 4

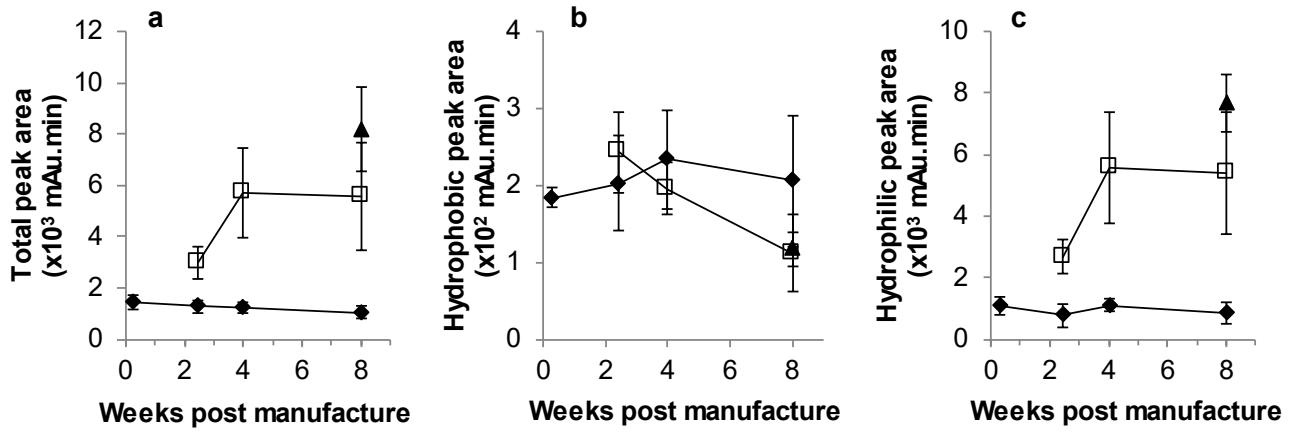


Figure 5

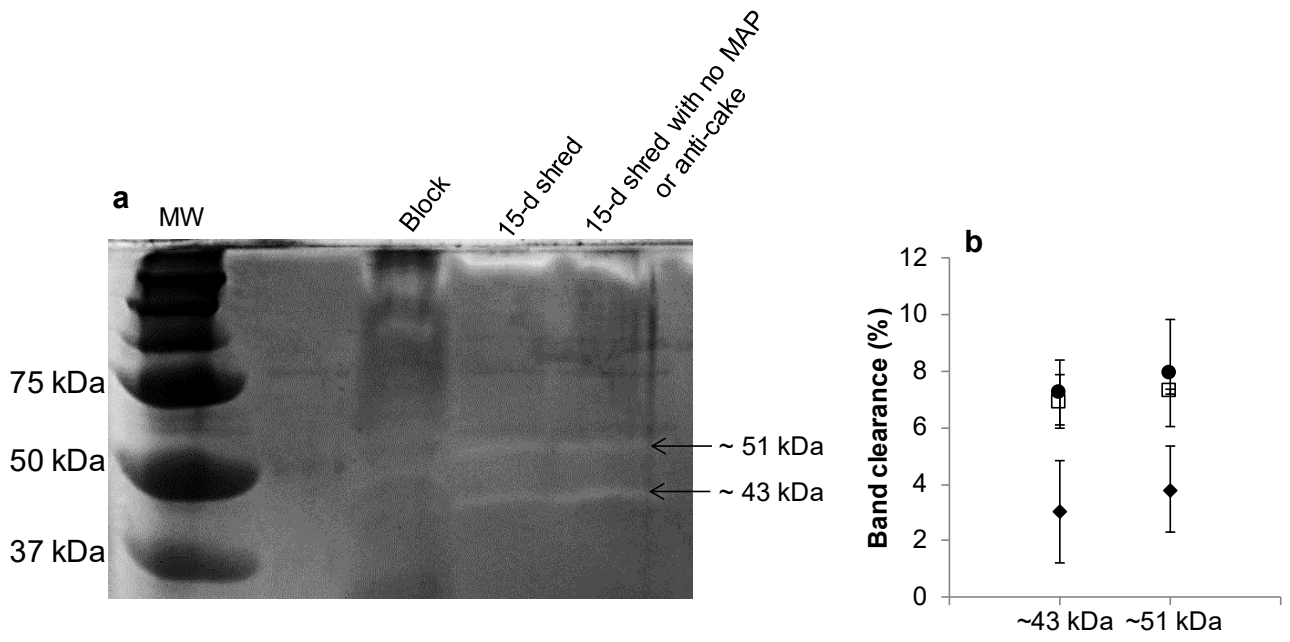


Figure 6

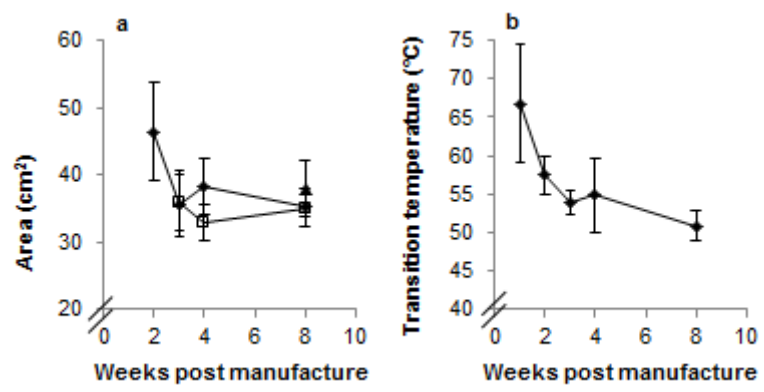


Figure 7

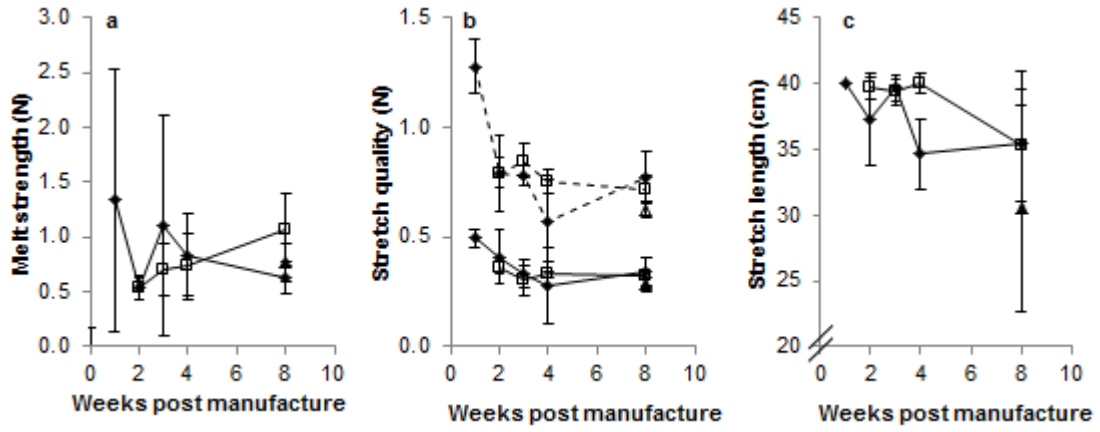


Figure 8

