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TITLE: Partitioning of starter bacteria and added exogenous enzyme activities between curd and whey during Cheddar cheese manufacture

AUTHOR(S): I.A. Doolan, A.B. Nongonierma, K.N. Kilcawley, M.G. Wilkinson

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42 **Partitioning of starter bacteria and added exogenous enzyme activities between**
43 **curd and whey during Cheddar cheese manufacture.**

44

45 **I. A. Doolan¹, A. B. Nongonierma², K. N. Kilcawley² and M. G. Wilkinson^{1*}**

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47 ¹ Department of Life Sciences, University of Limerick, Castletroy, Limerick, Ireland

48 ² Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

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57 *Corresponding Author. Mailing address: Department of Life Sciences, University
58 of Limerick, Castletroy, Limerick, Ireland.

59 Phone: +353 (0) 61 213440. Fax: +353 (0) 61 331490.

60 E-mail: Martin.Wilkinson@ul.ie

61

62 **Abstract**

63 Partitioning of starter bacteria and enzyme activities were investigated at different
64 stages of Cheddar cheese manufacture using three exogenous commercial enzyme
65 preparations added to milk or at salting. These enzymes included: Accelase AM317,
66 Accelase AHC50, Accelerzyme CPG. Flow cytometric analysis indicated that
67 AHC50 or AM317 consisted of permeabilized or dead cells and contained a range of
68 enzyme activities. The CPG preparation only contained carboxypeptidase activity.
69 Approximately 90% of the starter bacteria cells partitioned with the curd at whey
70 drainage. However, key enzyme activities partitioned with the bulk whey in the range
71 of 22% to 90%. An increased level of enzyme partitioning with the curd was
72 observed for AHC50 which was added at salting indicating the mode of addition
73 influenced partitioning. These findings suggest that further scope exists to optimize
74 both bacterial and exogenous enzyme incorporation into cheese curd to accelerate
75 cheese ripening.

76

77 **1. Introduction**

78 During Cheddar cheese manufacture starter lactococci are added to the milk for
79 acidification and flavour development. The starter microflora of Cheddar contributes
80 to proteolysis through the action of cell-envelope proteinases (CEP) and released
81 intracellular peptidases. Other agents contributing to proteolysis include: residual
82 plasmin and chymosin activities, non-starter lactic acid bacteria (NSLAB) and
83 exogenous proteinase preparations (McSweeney, 2004). As proteolysis is a rate-
84 limiting step in ripening it is important to maximise the contribution of the various
85 proteolytic agents to positively influence flavour and texture development. In the case
86 of starter lactococci, it is important to incorporate the maximal number of cells within
87 the curd in order to provide the necessary balance of enzymes required for flavour
88 development (Sheehan, O’Cuinn, FitzGerald, & Wilkinson, 2006). In Cheddar cheese
89 manufacture, whey is separated from the curd. The pH of the curd at whey drainage
90 has a significant role in determining compositional factors such as calcium content
91 and retained chymosin activity. At this point in the manufacturing process a
92 partitioning of the starter microflora between the solid curd and the liquid bulk whey
93 also occurs. Despite the importance of this step in determining the degree of cell
94 incorporation, published data is generally limited to direct plate counts (Bergamini,
95 Hynes, Quiberoni, Suarez, & Zalazar, 2005; Dawson & Feagan, 1957; Jeanson, et al.,
96 2011; Romeih, Moe, & Skeie, 2012). More recently, emerging technologies such as
97 flow cytometry (FCM) have been used to gain an insight into various microbial sub-
98 populations of live, permeabilized and dead cells in cheese during ripening (Doolan &
99 Wilkinson, 2009; Kilcawley, Nongonierma, Hannon, Doolan, & Wilkinson, 2012;
100 Sheehan, O’Loughlin, O’Cuinn, FitzGerald, & Wilkinson, 2005). Hence it would
101 therefore be useful to utilise this technique to augment direct plate count data in any

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102 | new partitioning studies conducted during Cheddar cheese manufacture. Acceleration
103 | of ripening time and/or flavour modification in Cheddar cheese has been undertaken
104 | in a number of studies involving addition of exogenous proteolytic preparations either
105 | with cheesemilk or at the salting stage (El Soda & Pandian, 1991; Hannon, et al.,
106 | 2003; Kailasapathy & Lam, 2005; Madkor, Tong, & El Soda, 2000; Wilkinson,
107 | Guinee, O'Callaghan, & Fox, 1992). A key factor in accelerating proteolysis is
108 | ensuring maximum incorporation of starter lactococci and added exogenous enzymes
109 | into the curd on the day of manufacture. In the case of coagulant added to milk for
110 | cheese manufacture estimated losses in the whey at drainage are of the order 85-94%
111 | (Bansal, Fox, & McSweeney, 2007; Garnot, Molle, & Piot, 1987; Holmes, Duersch,
112 | & Ernstrom, 1977). Holmes, et al. (1977) reported distribution of milk clotting
113 | enzymes as; 83% in bulk whey and 17% in bulk curd while after overnight curd
114 | pressing only 6% of the original activity remained. However, such detailed studies
115 | are lacking in the case of added exogenous proteinases for accelerated ripening. This
116 | information would be of benefit to cheese manufacturers to better understand the
117 | degree of enzyme incorporation that occurs when enzymes are added with milk or to
118 | curd at salting. The objectives of this study were to investigate the partitioning of
119 | starter lactococci during normal cheese manufacture and added key activities in
120 | exogenous commercial enzyme preparations added to cheese milk or at salting.

121

122 | **2. Material and methods**

123 | **2.1. Cheese making strains and cheese manufacture**

124 | Control or experimental cheeses were manufactured in triplicate as described by
125 | Kilcawley, et al. (2012). In each trial, control cheeses or those with added exogenous
126 | enzyme preparations were rotated into different vats on each day of cheesemaking to

128 avoid any possible contribution from vat-related factors. Accelase AM317 (Danisco,
129 Dange-Saint-Romain, France) and Accelerzyme CPG (DSM Food Specialties,
130 B.V., Delft, The Netherlands) were added to cheese milk while Accelase AHC50
131 (Danisco) was added with the salt as described by Kilcawley et al., (2012).
132 At the following points during manufacture cheeses were sampled for microbiology,
133 flow cytometry (FCM), enzymology and proteolysis: cheesemilk after inoculation
134 (A1), bulk whey at drainage (B1), bulk curd at drainage (B2), whey after salting (C1),
135 curd after salting (C2) and whey after pressing (D1). Parameters also monitored
136 during manufacture included curd yield and total production time as an average of the
137 three trials.

138

139 **2.2. Microbiological analysis**

140 L-M17 agar was used to determine the plate counts of starter culture during
141 manufacture of the cheeses (Terzaghi & Sandine, 1975). Milk or whey samples were
142 analysed by directly diluting with maximum recovery diluent (MRD) while curd
143 samples were prepared by diluting 1/10 (w/v) with sterile tri-sodium citrate (2% w/v)
144 and homogenized for 5 min using a stomacher (Seward Medical, London, UK).
145 Microbiological analysis was carried out in duplicate at each sampling point and L-
146 M17 agar plates were incubated at 30°C for 3 days.

147

148 **2.3. Activities in exogenous proteinase preparations prior to cheese manufacture**

149 *2.3.1. Azocasein activity*

150 General endoproteinase activity in the commercial enzyme preparations was
151 determined using an azocasein assay (Kilcawley, Wilkinson, & Fox, 2002). The
152 enzyme preparations were appropriately diluted in 0.05 M phosphate buffer pH 7.0,

153 on a volume basis (v/v) for CPG, and on a weight basis (w/v) for both AM317 and
154 AHC50. Each assay was performed in quadruplicate. Activity was expressed as the
155 change in absorbance.min⁻¹.mg⁻¹ protein under the assay conditions.

156

157 2.3.2. *Peptidase activities*

158 Commercial enzyme preparations were analysed for a range of peptidase activities
159 using different 7-amino-4-methyl coumarin (AMC) substrates (Table 2) at pH 7.0
160 (0.05 M Tris-HCl) or pH 5.2 (0.05 M citrate phosphate buffer) by modifications of the
161 methods described by Habibi-Najafi and Lee (1994), Kilcawley, et al. (2002) and
162 Kilcawley, et al. (2012). The release of AMC was calculated by reference to the
163 standard curve where the fluorescence of the sample was converted to nmol of AMC.
164 Activity was expressed as nmol min⁻¹ .mg⁻¹ protein.

165

166 2.3.3. *Esterase activity*

167 Esterase activity was measured in triplicate at pH 7.0 in 50 mM phosphate buffer at
168 pH 5.0 in a 0.1 M trisodium citrate buffer using *p*-nitrophenyl butyrate as described
169 by Hickey, Kilcawley, Beresford, Sheehan & Wilkinson (2006). A unit (U) of
170 activity was defined as the amount of enzyme that released 1 mM of *p*-nitrophenol
171 min⁻¹ .mg⁻¹ protein under the assay conditions.

172

173 2.3.4. *Lipase activity*

174 Lipase activity was measured according to the method of Stead (1983) with the
175 following modifications: a volume of 180 µL of sample was used and 3 mL of either
176 0.1 M Tris-HCl, pH 8.0 or a 0.1 M trisodium citrate buffer, pH 5.0. The substrate
177 consisted of 300 µL of a 16.6 mM solution of 4-methylumbelliferyl oleate (Sigma

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179 Aldrich). The samples were incubated at 37°C and fluorescence was measured over a
180 30 min period at an excitation and emission wavelength of 325 and 450 nm,
181 respectively using a Varian Cary Eclipse with Pelletier (Varian, JVA Analytical Ltd.),
182 With each sample, a blank consisting of 0.1 M Tris-HCl at pH 8.0 was used instead of
183 the sample to take account of any background fluorescence. A standard curve
184 ($R^2=0.991$) was generated in the range 0 to 20 mM 4-methylumbelliferone (the
185 primary product of the enzymatic reaction) prepared in the same manner as the 4-
186 methylumbelliferyl oleate solution. Activity was expressed in units, where one unit
187 (U) equalled the activity required to produce 1 μM of 4-methylumbelliferone $\cdot\text{s}^{-1}\cdot\text{mg}^{-1}$
188 protein under the assay conditions.

189

190 2.3.5. Carboxypeptidase activity (Alanine substrate)

191 Carboxypeptidase was determined using a modification of the method of (Doi,
192 Shibata, & Matoba, 1981) where alanine (Ala) was released from the substrate Z-Phe-
193 Ala (Bachem, Feinchemikalien, Bubendorf, Switzerland). Alanine released formed a
194 complex with cadmium-ninhydrin with an absorbance maximum at 506 nm. An
195 increase in absorbance was quantified from a standard curve produced using
196 concentrations of alanine (in the range 0.1, 0.2, 0.5, 0.8, 1.0 to 2.0 mM; $R^2=0.997$)
197 added to a test mixture of cadmium-ninhydrin. Absorbance at 506 nm was detected
198 using a Varian Cary Bio 100 spectrophotometer (JVA Analytical Ltd).

199 Carboxypeptidase activity was expressed as mM of Ala released. $\text{min}^{-1}\cdot\text{mg}^{-1}$ protein
200 and was determined at pH 5.0 (0.1 M trisodium citrate buffer) or pH 7.0 (0.05 M Tris-
201 HCl).

202

203 2.3.6. Carboxypeptidase activity (Leucine substrate).

204 Carboxypeptidase activity was measured at pH 3.2 and pH 5.0 (0.1 M trisodium
205 citrate buffer) and pH 7.0 (0.05 M Tris-HCl) using a modification of the method of
206 Hurley, O'Driscoll, Kelly, and McSweeney (1999) as described by Kilcawley, et al.
207 (2012). Carboxypeptidase activity was expressed as nM of heptapeptide degraded.
208 min⁻¹ .mg⁻¹ protein under the assay conditions.

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210 **2.4. Enzyme activities in the bulk whey and curd during cheese manufacture**

211 *2.4.1. Preparation of the curd extracts and whey samples*

212 Curd extracts were analysed during manufacture for various enzyme activities. Fresh
213 curd (20 g) collected on the day of production was mixed with 40 mL of 0.05 M
214 potassium phosphate buffer at pH 7.0 in a sterile stomacher bag. The sample was
215 homogenised in a Stomacher (IUL masticator, Lennox Laboratory Supplies Ltd,
216 Dublin, Ireland) for 5 min or until completely homogenous. A 10 mL aliquot of the
217 mixture was centrifuged at 4°C for 10 min at 10,000 g (Sorvall 5U centrifuge,
218 Unitech, Dublin, Ireland). Then 1 mL of the resultant supernatant was added to an
219 eppendorf tube and centrifuged at 13,000 g for 5 min (Eppendorf 5417C, VWR
220 International, Dublin, Ireland). The final supernatant was diluted as required and
221 subsequently assayed for various enzyme activities described above. Whey samples
222 were directly diluted using the appropriate buffer for the particular enzyme assay. All
223 enzyme assays were carried out in triplicate for each cheese trial on the three different
224 days of manufacture.

225 In the control cheese, Pep X or Pep N activities were monitored at all six sampling
226 points (A1, B1, B2, C1, C2 and D1) while all other enzyme activities were determined
227 in the bulk whey and bulk curd (B1 and B2) only. In experimental cheeses, enzyme
228 activities were monitored in the whey or curds after the addition of the exogenous

230 enzymes. For cheeses made with added AM317 or CPG, enzyme activities were
231 monitored in the bulk curd and bulk whey at drainage (B1 and B2, respectively). In
232 the case of cheeses with added AHC50 this preparation was added at salting and
233 consequently activities were monitored in the salted curd and salted whey (C1 and C2,
234 respectively).

235

236 *2.4.2. Enzyme activities in curd extracts and whey samples on day of manufacture*

237 | Enzyme activities present in the curd and whey samples were measured as per the
238 | methods described in section 2.3. In order to determine the partitioning of these
239 | enzymatic activities to the curd and whey respectively, the total volumes of the bulk
240 | curd, bulk whey, salted curd and salted whey were weighed (kg) and activities present
241 | in each sample was determined as a percentage of total activity. The total enzyme
242 | activity in the curd and whey in each cheese was calculated using the average
243 | volumes of curd and whey weighed on each day of manufacture. These are detailed
244 | in Table 1.

245

246 **2.5. Flow Cytometry of curd and whey samples during cheese manufacture**

247 Prior to cytometric analysis, whey or milk samples were diluted appropriately using
248 0.05 M potassium phosphate buffer pH 7.0 and immediately stained and analysed as
249 described by Kilcawley, et al. (2012).

250

251 **2.6. Free amino acid analysis**

252 Individual free amino acids (FAA) were determined on 24% TCA filtrates prepared
253 directly from the bulk whey or from a pH 4.6 water soluble nitrogen fraction of the
254 bulk curd as described by (Kuchroo & Fox, 1982). Duplicate samples were analysed

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256 using a Jeol JLC-500/V Amino Acid Analyzer (Jeol Ltd, Garden City, Herts, UK)
257 fitted with a Jeol sodium high performance cation exchange column. Results were
258 expressed as $\mu\text{g} \cdot \text{g}^{-1}$ cheese or whey.

259

260 **2.7. Statistical analysis**

261 Presentation of data was carried out using Microsoft® Excel. For the determination
262 of the accelerated ripening preparations enzyme activities, the experimental design
263 was a 3×2 factorial design, enzyme \times pH. A 2 way ANOVA followed by a Student
264 Newman-Keuls post-hoc test ($P < 0.05$) was carried out with SPSS (version 9, SPSS
265 Inc., Chicago, IL, USA). For the determination of the enzyme activities during cheese
266 production, 3 accelerated ripening preparations and one control were studied,
267 therefore a one way ANOVA was used for statistical analysis, followed by a Student
268 Newman-Keuls post-hoc test ($P < 0.05$) with SPSS. Two-way ANOVA analysis with
269 the Tukey's significance difference test ($P < 0.05$) was used for the analysis of FCM
270 data. R (2011) software was used for statistical analysis (Development Core Team,
271 2011).

272

273 **3. Results and Discussion**

274 **3.1. Enzyme activities of the accelerated ripening preparations**

275 Peptidase activities detected in the three exogenous commercial preparations are
276 presented in Table 1. Of the twelve substrates examined, substantial Pep X activity
277 was detected in both AM317 and AHC50 preparations and was higher at pH 7.0
278 compared with pH 5.2 for both preparations. In contrast, Accelerzyme CPG
279 contained very little activity against the range of substrates tested, except for Pep R
280 which was detectable only at pH 5.2 (Table 1). Both AHC50 and AM317

281 preparations had similar levels of activity towards the Z-Phe-Ala substrate which were
282 significantly higher ($P<0.05$) at pH 7.0 (Table 2). The CPG preparation displayed
283 little activity against the Z-Phe-Ala substrate and could only release low levels of
284 alanine from this peptide. However, CPG displayed the highest activity of the three
285 enzyme preparations against the heptapeptide substrate Pro-Thr-Glu-Phe-[NO₂-Phe]-
286 Arg-Leu, which may indicate carboxypeptidase activity (Table 2). This activity was
287 ~10 fold greater for the CPG preparation compared with AM317 or AHC50 at pH 3.2
288 and 5.0, but was similar to the other two commercial enzyme preparations at pH 7.0
289 (Table 2).

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290 Overall, proteolytic activities for each preparation towards the azocasein substrate at
291 pH 5.0 or 7.0 were always significantly higher ($P<0.05$) for AM317 than the AHC50
292 preparation, with no activity detected for the CPG preparation (Table 3). For all three
293 enzyme preparations, esterase activity was significantly higher in both AM317 and
294 AHC50 preparations. This activity was also significantly higher ($P<0.05$) at pH 5.0
295 compared with pH 7.0 (Table 2). Lipase activity was significantly higher ($P<0.05$) in
296 AM317 compared with the two other preparations and, in contrast to esterase activity,
297 was highest at pH 5.0 for the latter enzyme preparation (Table 2). These results
298 highlight a major difference in the range of enzyme activities contained in the CPG
299 preparation in comparison to AM317 or AHC50 preparations.

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301 3.2. Cheese manufacturing

302 Cheeses manufactured with added AM317 had coagulation times of 36.33 min
303 compared with control cheeses (40.00 min), while cheeses made with CPG or AHC50
304 had somewhat extended coagulation times of 43.67 and 46.67 min, respectively.

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305 Total curd yields for all cheeses ranged from 47.3 to 49.5 kg per 454 L of cheese

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310 | milk. Overall, Control or AM317 cheeses had the shortest manufacturing times, but
311 | all cheeses were manufactured within times (from addition of starter until pre-press)
312 | ranging from 328 to 343 min (data not shown), which were not statistically significant
313 | ($P \geq 0.05$).

314

315 | **3.3. Microbiological analysis**

316 | The viable starter counts on L-M17 agar for all cheeses at each sampling point over
317 | triplicate manufacturing trials were calculated. Overall, an increase in cell numbers
318 | was observed in the bulk curd samples for all cheeses corresponding to cell growth
319 | and pH development. For all cheeses an increase in starter populations was observed
320 | between the bulk curd after drainage and the salted curd. The only statistically
321 | significant difference ($P < 0.05$) in LAB starter counts was found in the bulk curd
322 | samples at whey drainage where higher populations were noted for cheese with added
323 | AM317 compared with cheese with added CPG preparation.

324 | The calculated percentages of starter bacteria partitioning between the bulk curd and
325 | bulk whey and subsequently between the salted curd and the salted whey are shown in
326 | Fig. 1. The calculated percentage retention of starter bacteria in the bulk curd for
327 | Control or AHC50 cheeses was 94.7% or 91.9%, respectively. In contrast, retention
328 | of starter bacteria in AM317 or CPG-treated cheeses was 88.8% or 81.5%,
329 | respectively (Fig. 1a). This finding appears in general agreement with that reported
330 | by (Jeanson, et al., 2011) who stated that ~90%, of starter cells preferentially partition
331 | with the curd. These workers also highlighted the importance of final curd numbers
332 | in terms of spatial distribution and possible influences on diffusion of solutes and
333 | enzymes to and from starter cells during ripening. It has been postulated by Laloy,
334 | Vuilleumard, El Soda, and Simard (1996) that bacteria may partition with the curd

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337 | based on an affinity for the milk fat globule membrane. At salting an average ~99%
338 | retention of starter was calculated for all cheeses with ~1% losses of cells in the salted
339 | whey (Fig. 1b).

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341 | **3.4. Pep X and Pep N activities during cheese production**

342 | Pep X and Pep N activities of AHC50-treated cheese were considered to be similar to
343 | the control as this preparation was not added to the cheesemilk. Activity of Pep X or

344 | Pep N detected in Control samples may have originated from early autolysis of starter

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345 | culture during cheese production (Table 33). For all cheeses, Pep X activity was
346 | significantly ($P < 0.05$) higher than Pep N activity in the bulk curd and bulk whey.

347 | Additionally, Pep X activity in the bulk curd was not significantly different ($P \geq 0.05$)

348 | between any of the cheeses and the calculated partitioning to the bulk whey was

349 | similar (~67%) for all cheeses. For Pep N activity, 21 or 35% partitioned with the

350 | bulk whey for AM317 or CPG treated cheeses, respectively. The differences in

351 | enzyme partitioning to the bulk whey observed between Pep X and Pep N may be due

352 | to differences in enzyme stability, conformation, affinity with the cheese matrix and

353 | accessibility of required co-factors.

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354 | No significant differences ($P \geq 0.05$) in Pep N activity were observed in bulk whey or

355 | bulk curd between any of the cheeses (Table 3). At salting, partitioning of Pep X and

356 | Pep N to the whey in the Control cheese was relatively low (<2%, Table 3). In the

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357 | cheeses with added AHC50, the levels of Pep X in the salted curd was significantly

358 | higher ($P < 0.05$) when compared with control cheeses. In AHC50-treated cheeses,

359 | 95.1 or 90.3% of Pep X and Pep N partitioned with the salted curd, respectively.

360 | Partitioning of these activities with the salted whey/ pressed whey were lower for

361 | AHC50 cheese compared with AM317 or CPG-treated cheeses. Overall this data

367 supports the general idea that addition of enzyme preparation with salt can reduce
368 partitioning of enzyme with the whey (Wilkinson & Kilcawley, 2005). The higher
369 Pep X levels seen in the salted whey of AHC50 cheeses may be attributed to the mode
370 of addition of the AHC50 preparation which was added to the salt.

371

372 **3.5. Other enzyme activities (carboxypeptidase, esterase and lipase) in the whey** 373 **and curd**

374 In all cheeses a comparison of other enzyme activities monitored indicated no
375 significant difference ($P \geq 0.05$) in activities partitioning with the bulk curd or bulk
376 whey (Table 4). Significantly higher lipase activity was found in the bulk curd
377 compared to the bulk whey (Table 5). Carboxypeptidase and esterase activities were
378 not significantly higher ($P \geq 0.05$) in the bulk whey compared to the bulk curd, except
379 for carboxypeptidase activity against Leucine substrate in AM317-treated cheeses
380 (Table 4). Lipase activities displayed the opposite trend with higher activity
381 partitioning with the bulk curd ($P < 0.05$). Partitioning of carboxypeptidases and
382 esterases activities with bulk whey was relatively high ranging from 67.3 to 90.5 %.
383 These values are in accordance with Wilkinson and Kilcawley (2005) who reviewed
384 the area and noted that some studies reported partitioning of enzymes to the bulk
385 whey at drainage may be as high as 90%. Partitioning of lipase activity with the whey
386 was lower with values ranging from 44.2 to 49.7%.

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388 **3.6 Flow cytometric analysis of starter cells on day of manufacture**

389 Dot plots were generated at all sampling points for each of the cheeses and data were
390 expressed as a percentage of live, permeabilized or dead cells (Fig. 2). Significant
391 differences ($P \geq 0.05$) in live cells were not evident between cheeses at any sampling

393 point (Table 5). However, in the AHC50-treated cheeses levels of permeabilized or
394 dead cells were significantly different ($P<0.05$) in the salted whey and pressed whey
395 in comparison to the other cheeses. Previous FCM data found that the AHC50
396 preparation contained primarily permeabilized/dead cells (Kilcawley, et al., 2012).
397 These cells may have been generated following an attenuation treatment as the
398 cytometric profiles were similar to those obtained following CTAB treatment of
399 lactococcal strains observed by Doolan and Wilkinson (2009) and Sheehan, et al.
400 (2005). Thus, it appears that most of the permeabilized/dead cells in the AHC50
401 preparation were lost to the salted and pressed whey immediately after addition. It is
402 possible that in the case of AHC 50, the original cells may have been exposed to
403 treatments which may have removed cell surface components associated with
404 electrostatic attraction and incorporation into the curd matrix. Indeed, Crow, Gopal,
405 and Wicken (1995) noted that treatments which altered the cell surface components of
406 a range of starter strains but which did not cause autolysis resulted in differing
407 degrees of hydrophobicity and phage adsorption. While these authors did not link
408 these effects with cell partitioning in cheese manufacture, it is reasonable to postulate
409 that the type of permeabilising treatments used for AHC 50 manufacture may have
410 also impacted on cell surface architecture and partitioning properties. Doolan and
411 Wilkinson (2009) noted that significant treatment-related effects on cell permeability
412 and intracellular enzyme release, however in the case of AHC 50 this information is
413 not available. Therefore while an industrial process may have been optimised to
414 enhance cell permeability and intracellular enzyme release it may have adversely
415 affected cell surface properties and consequent retention during cheese manufacture.

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417 3.7. Free amino acid analysis

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423 Total FAA levels were significantly ($P < 0.05$) higher in the bulk whey of the AM317-
424 treated cheeses compared to the other cheeses and was more than double that of the
425 other cheeses (Fig. 3). The individual FAA profile of the bulk whey of the AM317
426 cheeses was also very different to that of the other cheeses. However, Pep X and Pep
427 N activities in the bulk whey of the AM317-treated cheeses were not significantly
428 ($P \geq 0.05$) higher than in the other cheeses. Hence differences in total FAA levels may
429 be due to higher levels of proteolytic activity contained in the AM317 preparation
430 (Table 2) and which may have a different substrate specificity compared with
431 chymosin. Such enzymatic differences could account for differences in FFA levels
432 generated and in individual FAA profiles. As the CPG preparation did not contain
433 any proteinase activity and the AHC50 preparation was added with the salt, levels of
434 FAA in the bulk whey from both these cheeses should not differ from each other in
435 the bulk whey. Although the additional losses of FAA in the bulk whey of AM317
436 cheeses did not impact on yield it may impact on the potential downstream processing
437 applications for this whey. The extent of partitioning with the bulk whey found for
438 commercial enzymes represents an adverse economic outcome in terms of cost
439 benefits to cheese manufacturers. Therefore further optimisation of methods for the
440 addition of exogenous enzyme preparations in cheese manufacture are still required to
441 achieve the most cost effective outcomes.

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443 **4.0 Conclusion**

444 In this study, cheese was produced using commercial enzyme preparations marketed
445 as accelerated ripening agents. Pre-screening of these preparations prior to their use
446 identified CPG as a relatively pure preparation with minimal enzymatic side enzyme
447 activities present. However, both the AM317 and AHC50 preparations contained a

450 range of enzymatic activities and previous studies by Kilcawley, et al. (2012) have
451 shown that these preparations consist primarily of permeabilized and dead bacterial
452 cells. During Cheddar cheese production, approximately 90% of the starter cells
453 partitioned with the bulk curd after whey drainage. Significant partitioning of enzyme
454 activities with the bulk whey was observed ranging from 22 to 90% of the added
455 initial levels. Lowest partitioning with the whey was noted for Pep N activity and
456 highest for esterase and carboxypeptidase activities. In the cheese where the enzyme
457 preparation was added with the salt (AHC50), partitioning of Pep X and Pep N
458 activity to the salted curd was 95.2 and 90.3%, respectively, when the volumes of
459 salted whey and salted curd were taken into account. Overall it would appear that the
460 addition of exogenous enzyme preparations with the salt rather than to the cheesemilk
461 may significantly improve their retention. Free amino acid analysis of bulk whey
462 samples revealed high levels of FAA in the bulk whey from cheese treated with
463 AM317, which may impact on the suitability of such whey for certain applications.
464 This study has provided useful information on partitioning of both starter bacteria and
465 added exogenous enzymes in the curd and whey during manufacture of potential
466 interest to enzyme manufacturers and cheese producers alike.

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476 **Table 1.** Peptidase activities of the commercial accelerated ripening systems at pH 5.2 and 7.0. Values presented are the mean±SD of triplicate
 477 trials.
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Substrate	Peptidase	Activity (nmol min ⁻¹ mL ⁻¹) Mean±SD					
		AM317		Accelerzyme CPG		AHC50	
		pH 7.0	pH 5.2	pH 7.0	pH 5.2	pH 7.0	pH 5.2
Pep N	H-Lys-AMC	8.4±0.0	0.0±0.0	0.1±0.1	0.2±0.0	5.6±4.9	0.0±0.0
Pep X	H-Gly-Pro-AMC	940.1±79.5	587.33±44.86	0.0±0.0	0.3±0.0	1598.9±164.2	669.5±85.1
Pep A (1)	H-Asp-AMC	5.6±4.7	0.0±0.0	0.1±0.0	0.1±0.0	2.8±4.9	6.4±11.2
Pep A (2)	H-Glu-AMC	20.8±6.2	0.0±0.0	0.0±0.0	0.3±0.0	10.5±3.6	0.0±0.0
Pep M	H-Met-AMC	0.0±0.0	0.0±0.0	0.3±0.0	0.15±0.03	0.0±0.0	0.0±0.0
Pep R	Z-Pro-Arg-AMC-HCl	7.7±7.3	0.0±0.0	0.2±0.0	25.9±4.5	7.7±7.3	0.0±0.0
Pep Q	Z-Gly-Pro-AMC	14.6±6.2	0.0±0.0	0.0±0.0	0.3±0.0	0.0±0.0	15.4±3.4
Pep I	H-Pro-AMC	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.1	9.7±8.5	12.9±12.6

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482 **Table 2.** Carboxypeptidase, Esterase, Lipase and Azocasein activities of the commercial accelerated ripening systems at different pH values.
 483 Values presented are the mean±SD of triplicate trials.
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Enzyme assay	pH	Mean activity ±SD		
		AM317	Accelerzyme CPG	AHC50
Carboxypeptidase- Ala substrate (mM/min/mg)	5.0	251.0±21.7 ^b	14.7±1.4 ^a	235.3±11.3 ^b
	7.0	531.1±4.5 ^c	19.9±0.5 ^a	521.8±31.6 ^c
Carboxypeptidase- Leu substrate (mM/min/mg)	3.2	4.0±0.1 ^c	39.7±0.1 ^c	1.6±0.1 ^a
	5.0	3.1±0.1 ^b	39.7±0.1 ^c	1.9±0.4 ^a
	7.0	4.8±0.1 ^d	5.7±0.8 ^d	5.4±0.1 ^d
Esterase (µM/min)	5.0	1949.7±10.7 ^d	209.5±19.7 ^c	1914.8±32.7 ^d
	7.0	122.7±8.3 ^b	24.6±1.7 ^a	50.4±8.6 ^a
Lipase (pM/s/mg)	5.0	44.0±13.5 ^c	0.3±0.1 ^a	1.2±0.2 ^a
	8.0	26.7±3.8 ^b	0.3±0.1 ^a	1.4±0.2 ^a
Azocasein (OD/min/mg)	5.0	0.8±0.1 ^b	nd	0.3±0.1 ^a
	7.0	2.8±0.1 ^d	nd	1.2±0.1 ^c

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491 **Table 3.** Pep X and Pep N activities (per mL of whey or per g of curd) during production (\pm SD of triplicate trials). Bulk whey (B1), bulk curd
 492 (B2), salted whey (C1) and salted curd (C2)

	Pep N activity			Pep X activity			Pep N activity			Pep X activity		
	B1*	B2*	Partition to the whey%	B1*	B2*	Partition to the whey%	C1*	C2*	Partition to the whey%	C1*	C2*	Partition to the whey%
Control	0.00 \pm 0.0 ^a	0.14 \pm 0.089 ^a	0	0.43 \pm 0.03 ^a	1.68 \pm 0.36 ^a	68.2	0.63 \pm 0.06 ^a	0.25 \pm 0.08 ^a	1.48	0.45 \pm 0.08 ^a	3.53 \pm 1.00 ^a	0.16
AM317	0.00 \pm 0.0 ^a	0.09 \pm 0.11 ^a	21.4	0.46 \pm 0.09 ^a	1.92 \pm 0.83 ^a	67.4	nd	nd	nd	nd	nd	nd
CPG	0.00 \pm 0.0 ^a	0.06 \pm 0.06 ^a	35.3	0.42 \pm 0.10 ^a	1.75 \pm 1.04 ^a	66.8	nd	nd	nd	nd	nd	nd
AHC50	nd	nd	nd	nd	nd	nd	4.60 \pm 4.75 ^a	0.53 \pm 0.46	9.7	1.76 \pm 1.33 ^a	1.28 \pm 0.39 ^b	4.9

493 Values presented are the mean \pm SD of triplicate trials
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495 n.d.: not determined

496 *In the same column, figures with the same letter are not significantly different ($P \geq 0.05$)

497 Abbreviations: Pep N, aminopeptidase N; Pep X, Post-prolyl di-peptidyl aminopeptidase X; B1, Bulk whey; B2, Bulk Curd, Activity; Enzyme activity; Partition to the whey, % enzyme partitioned to the whey.
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499 **Table 4.** Carboxypeptidase, chymosin, esterase, and lipase activity (\pm SD of triplicate trials) in the bulk whey (B1) and bulk curd (B2) of the
 500 control, CPG and AM317 cheeses. Enzyme activities are expressed per mL of whey or per g of curd.

	Carboxypeptidase (Ala substrate)			Carboxypeptidase (Leu substrate)			Esterase			Lipase		
	Activity (mM/min)		Partition to the whey (%)	Activity (nM/min)		Partition to the whey (%)	Activity (nM/min)		Partition to the whey (%)	Activity (pM/s)		Partition to the whey (%)
	B1*	B2*		B1*	B2*		B1*	B2*		B1*	B2*	
Control	0.06 \pm 0.01 ^a	0.04 \pm 0.01 ^a	90.5	7.6 \pm 2.0 ^a	14.8 \pm 13.4 ^{a,b}	81.2	100.6 \pm 39.4 ^a	138.0 \pm 136.9 ^a	67.2	65.6 \pm 13.8 ^a	559.5 \pm 434.6 ^b	49.7
CPG	0.06 \pm 0.02 ^a	0.05 \pm 0.03 ^a	84.1	n.d.	n.d.	n.d.	127.4 \pm 19.3 ^a	150.8 \pm 128.5 ^a	70.3	64.9 \pm 7.8 ^a	592.5 \pm 191.2 ^b	48.1
AM317	0.08 \pm 0.02 ^a	0.06 \pm 0.03 ^a	86.2	9.1 \pm 2.9 ^a	21.3 \pm 1.0 ^b	77.8	n.d.	n.d.	n.d.	65.2 \pm 5.8 ^a	669.1 \pm 278.5 ^b	44.2

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Values presented are the mean \pm SD of triplicate trials

n.d.: not determined

*Within the same enzyme activity, figures with the same letter are not significantly different ($P \geq 0.05$)

Abbreviations: Ala substrate, Z-Phe-Ala; Leu substrate, Pro-Thr-Glu-Phe-[NO₂-Phe]-Arg-Leu; Activity, enzyme activity; B1, Bulk Whey; B2, Bulk Curd; Partition to the whey, % enzyme partitioned to the whey.

506 **Table 5.** Percentage of cells detected by Flow Cytometry for each of the regions: live, permeabilized, or dead for control or experimental
 507 cheeses at each of the sampling points: After inoculation (A1), Bulk Whey (B1), Bulk Curd (B2), Salted whey (C1), Salted curd (C2) and
 508 pressed whey (D1).
 509

Region	Cheese	A1*	B1*	B2*	C1*	C2*	D1*
Live	CTL	26.6±7.7 ^a	28.0±13.2 ^a	17.2±6.3 ^a	29.2±7.8 ^a	17.3±7.1 ^a	37.3±19.7 ^a
	AM317	22.0±12.1 ^a	23.5±11.6 ^a	24.6±15.5 ^a	33.5±11.8 ^a	14.1±6.2 ^a	28.1±13.6 ^a
	CPG	22.2±13.6 ^a	21.3±12.7 ^a	15.2±5.2 ^a	34.5±22.2 ^a	13.5±5.4 ^a	35.3±22.4 ^a
	AHC50	18.3±8.0 ^a	23.9±13.6 ^a	16.0±5.1 ^a	7.1±3.1 ^b	15.1±7.6 ^a	11.3±6.4 ^a
Permeabilized	CTL	26.0±0.6 ^a	12.9±1.4 ^a	23.9±5.6 ^a	14.5±1.6 ^a	21.2±5.8 ^a	12.7±1.0 ^a
	AM317	26.3±7.8 ^a	13.2±3.9 ^a	37.1±16.1 ^a	14.1±2.3 ^a	24.2±9.9 ^a	14.3±7.0 ^a
	CPG	22.4±2.5 ^a	14.3±2.8 ^a	29.8±11.2 ^a	12.7±1.3 ^a	33.0±15.6 ^a	12.9±3.0 ^a
	AHC50	25.4±8.0 ^a	13.9±0.9 ^a	31.2±7.8 ^a	5.8±2.2 ^b	31.0±5.6 ^a	5.9±1.3 ^b
Dead	CTL	44.5±8.6 ^a	63.6±12.1 ^a	58.5±9.0 ^a	53.9±9.0 ^a	62.1±11.8 ^a	55.6±17.9 ^a
	AM317	62.3±15.2 ^a	49.1±20.20 ^a	45.6±30.4 ^a	52.9±10.5 ^a	62.2±12.8 ^a	57.5±13.0 ^a
	CPG	54.1±10.9 ^a	64.8±8.9 ^a	54.8±13.5 ^a	50.6±19.1 ^a	52.2±18.4 ^a	48.7±17.9 ^a
	AHC50	46.6±20.4 ^a	63.8±10.0 ^a	53.9±10.7 ^a	80.2±4.70 ^b	53.9±10.0 ^a	84.4±5.4 ^b

510 Values presented are the mean±SD of triplicate trials
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512 *Within the same column, figures with the same letter are not significantly different ($P \geq 0.05$)

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