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

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Abstract

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

1. Introduction

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

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

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2. Material and methods

2.1. Cheese making strains and cheese manufacture

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

avoid any possible contribution from vat-related factors. Accelase AM317 (Danisco, Dange-Saint-Romain, France) and Accelerzyme CPG (DSM Food Specialties, B.V,Delft, The Netherlands) were added to cheese milk while Accelase AHC50 (Danisco) was added with the salt as described by Kilcawley et al., (2012).

At the following points during manufacture cheeses were sampled for microbiology, flow cytometry (FCM), enzymology and proteolysis: cheesemilk after inoculation (A1), bulk whey at drainage (B1), bulk curd at drainage (B2), whey after salting (C1), curd after salting (C2) and whey after pressing (D1). Parameters also monitored during manufacture included curd yield and total production time as an average of the three trials.

2.2. Microbiological analysis

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

2.3. Activities in exogenous proteinase preparations prior to cheese manufacture

2.3.1. Azocasein activity

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

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

2.3.2. Peptidase activities

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

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2.3.3. Esterase activity

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

2.3.4. Lipase activity

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

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

2.3.5. Carboxypeptidase activity (Alanine substrate)

Carboxypeptidase was determined using a modification of the method of (Doi, Shibata, & Matoba, 1981) where alanine (Ala) was released from the substrate Z-Phe-Ala (Bachem, Feinchemikalien, Bubendorf, Switzerland). Alanine released formed a complex with cadmium-ninhydrin with an absorbance maximum at 506 nm. An increase in absorbance was quantified from a standard curve produced using concentrations of alanine (in the range 0.1, 0.2, 0.5, 0.8, 1.0 to 2.0 mM; R^2=0.997) added to a test mixture of cadmium-ninhydrin. Absorbance at 506 nm was detected using a Varian Cary Bio 100 spectrophotometer (JVA Analytical Ltd). Carboxypeptidase activity was expressed as mM of Ala released. min^{-1} $.mg^{-1}$ protein and was determined at pH 5.0 (0.1 M trisodium citrate buffer) or pH 7.0 (0.05 M Tris-HCl).

2.3.6. Carboxypeptidase activity (Leucine substrate).

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

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

2.4.1. Preparation of the curd extracts and whey samples

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

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

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

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

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

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

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

2.6. Free amino acid analysis

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

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

2.7. Statistical analysis

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

3. Results and Discussion

3.1. Enzyme activities of the accelerated ripening preparations

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

preparations had similar levels of activity towards the Z-Phe-Ala substrate which were significantly higher ($P<0.05$) at pH 7.0 (Table 2). The CPG preparation displayed little activity against the Z-Phe-Ala substrate and could only release low levels of alanine from this peptide. However, CPG displayed the highest activity of the three enzyme preparations against the heptapeptide substrate Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu, which may indicate carboxypeptidase activity (Table 2). This activity was ~10 fold greater for the CPG preparation compared with AM317 or AHC50 at pH 3.2 and 5.0, but was similar to the other two commercial enzyme preparations at pH 7.0 (Table 2).

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

3.2. Cheese manufacturing

Cheeses manufactured with added AM317 had coagulation times of 36.33 min compared with control cheeses (40.00 min), while cheeses made with CPG or AHC50 had somewhat extended coagulation times of 43.67 and 46.67 min, respectively. Total curd yields for all cheeses ranged from 47.3 to 49.5 kg per 454 L of cheese

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

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3.3. Microbiological analysis

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

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

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

3.4. Pep X and Pep N activities during cheese production

Pep X and Pep N activities of AHC50-treated cheese were considered to be similar to the control as this preparation was not added to the cheesemilk. Activity of Pep X or Pep N detected in Control samples may have originated from early autolysis of starter culture during cheese production (Table 33). For all cheeses, Pep X activity was significantly (P<0.05) higher than Pep N activity in the bulk curd and bulk whey. Additionally, Pep X activity in the bulk curd was not significantly different (P≥0.05) between any of the cheeses and the calculated partitioning to the bulk whey was similar (~67%) for all cheeses. For Pep N activity, 21 or 35% partitioned with the bulk whey for AM317 or CPG treated cheeses, respectively. The differences in enzyme partitioning to the bulk whey observed between Pep X and Pep N may be due to differences in enzyme stability, conformation, affinity with the cheese matrix and accessibility of required co-factors.

No significant differences (P≥0.05) in Pep N activity were observed in bulk whey or bulk curd between any of the cheeses (Table 3). At salting, partitioning of Pep X and Pep N to the whey in the Control cheese was relatively low (<2%, Table 3). In the cheeses with added AHC50, the levels of Pep X in the salted curd was significantly higher (P<0.05) when compared with control cheeses. In AHC50-treated cheeses, 95.1 or 90.3% of Pep X and Pep N partitioned with the salted curd, respectively. Partitioning of these activities with the salted whey/ pressed whey were lower for AHC50 cheese compared with AM317 or CPG-treated cheeses. Overall this data

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

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

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

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3.6 Flow cytometric analysis of starter cells on day of manufacture

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

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

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3.7. Free amino acid analysis

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Total FAA levels were significantly ($P<0.05$) higher in the bulk whey of the AM317-treated cheeses compared to the other cheeses and was more than double that of the other cheeses (Fig. 3). The individual FAA profile of the bulk whey of the AM317 cheeses was also very different to that of the other cheeses. However, Pep X and Pep N activities in the bulk whey of the AM317-treated cheeses were not significantly ($P\geq0.05$) higher than in the other cheeses. Hence differences in total FAA levels may be due to higher levels of proteolytic activity contained in the AM317 preparation (Table 2) and which may have a different substrate specificity compared with chymosin. Such enzymatic differences could account for differences in FFA levels generated and in individual FAA profiles. As the CPG preparation did not contain any proteinase activity and the AHC50 preparation was added with the salt, levels of FAA in the bulk whey from both these cheeses should not differ from each other in the bulk whey. Although the additional losses of FAA in the bulk whey of AM317 cheeses did not impact on yield it may impact on the potential downstream processing applications for this whey. The extent of partitioning with the bulk whey found for commercial enzymes represents an adverse economic outcome in terms of cost benefits to cheese manufacturers. Therefore further optimisation of methods for the addition of exogenous enzyme preparations in cheese manufacture are still required to achieve the most cost effective outcomes.

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4.0 Conclusion

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

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

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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 trials.

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

Table 2. Carboxypeptidase, Esterase, Lipase and Azocasein activities of the commercial accelerated ripening systems at different pH values. Values presented are the mean±SD of triplicate trials.

		Mean activity ±SD		
Enzyme assay	**pH**	**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^{e}	1.6±0.1^{a}
	5.0	3.1±0.1^{b}	39.7±0.1^{e}	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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Table 3. Pep X and Pep N activities (per mL of whey or per g of curd) during production (±SD of triplicate trials)**.** Bulk whey (B1), bulk curd (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±0.0[a]	0.14±0.089[a]	0	0.43±0.03[a]	1.68±0.36[a]	68.2	0.63±0.06[a]	0.25±0.08[a]	1.48	0.45±0.08[a]	3.53±1.00[a]	0.16
AM317	0.00±0.0[a]	0.09±0.11[a]	21.4	0.46±0.09[a]	1.92±0.83[a]	67.4	nd	nd	nd	nd	nd	nd
CPG	0.00±0.0[a]	0.06±0.06[a]	35.3	0.42±0.10[a]	1.75±1.04[a]	66.8	nd	nd	nd	nd	nd	nd
AHC50	nd	nd	nd	nd	nd	nd	4.60±4.75[a]	0.53±0.46	9.7	1.76±1.33[a]	1.28±0.39[b]	4.9

Values presented are the mean±SD of triplicate trials

n.d.: not determined

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

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.

Table 4. Carboxypeptidase, chymosin, esterase, and lipase activity (±SD of triplicate trials) in the bulk whey (B1) and bulk curd (B2) of the 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±0.01[a]	0.04±0.01[a]	90.5	7.6±2.0[a]	14.8±13.4[a,b]	81.2	100.6±39.4[a]	138.0±136.9[a]	67.2	65.6±13.8[a]	559.5 ± 434.6[b]	49.7
CPG	0.06±0.02[a]	0.05±0.03[a]	84.1	n.d.	n.d.	n.d.	127.4±19.3[a]	150.8±128.5[a]	70.3	64.9±7.8[a]	592.5±191.2[b]	48.1
AM317	0.08±0.02[a]	0.06±0.03[a]	86.2	9.1±2.9[a]	21.3±1.0[b]	77.8	n.d.	n.d.	n.d.	65.2±5.8[a]	669.1±278.5[b]	44.2

Values presented are the mean±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-[NO_2-Phe]-Arg-Leu; Activity, enzyme activity; B1, Bulk Whey; B2, Bulk Curd; Partition to the whey , % enzyme partitioned to the whey.

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

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]

Values presented are the mean±SD of triplicate trials

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

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