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Peptidolytic, esterolytic and amino acid catabolic activities of selected bacterial strains from the surface of smear cheese

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Abstract

Enzymes produced by bacteria present on the surface of smear cheeses play essential roles in flavour development during cheese ripening. In this study, strains including brevibacteria, corynebacteria, staphylococci and brachybacteria, from the surface of two smear cheese (Tilsit and Gubeen) were screened for a range of enzyme activities including aminopeptidase (substrates: Leu-pNA and His-pNA), dipeptidase (Met–Ala, Ala–Met, Pro–Ala, His–Leu and Pro–Leu), tripeptidase (Phe–Gly–Gly, Gly–Gly–Gly and Leu–Ala–Pro), esterase (β-naphthyl butyrate, β-naphthyl caprate and β-naphthyl palmitate), L-methionine aminotransferase and cystathionine lyase activities. There were marked differences in the activities observed between different bacteria studied. Brachybacteria showed low activity on all substrates assayed. There was no consistency in activities within groups of related bacteria. For example, *Staphylococcus equorum* 14 showed higher activity than *S. equorum* 6 on all the substrates tested. Among the corynebacteria, *Corynebacterium ammoniagenes* CA8 had greatest aminopeptidase, esterase and cystathionine lyase activity while *C. casei* B showed more di- and tri-peptidase activity. It was noted that individual bacteria displayed similar activities on all three esterase substrates, i.e., the chain length of the fatty acid did not appear to affect activity. L-Methionine aminotransferase activity was observed in only one strain (*S. equorum* 14) whereas all strains had cystathionine lyase activity.

Keywords: Smear cheeses; Cheese ripening

1. Introduction

Smear-ripened cheeses are those in which bacteria are present in large numbers on the surface of the cheese and make a significant contribution to its characteristic flavour, appearance and aroma. Examples of smear-ripened cheeses include Tilsit, Trappist, Limburger, Brick and Pont l'Eveque (Reps, 1993).

There are three main biochemical events in cheese ripening—proteolysis (widely considered the most important series of reactions), lipolysis and glycolysis (Fox, 1989). The roles of lactic acid bacteria and their enzymes in cheese ripening have been researched extensively, including their proteolytic systems (Tan et al., 1993; Bockelmann, 1995; Kunji et al., 1996; Law and Haandrikman, 1997). There has not been the same level of research on the bacteria associated with the surface of smear cheeses, so the contribution of these smear strains to cheese ripening is less clear. It is known that glycolysis is largely completed by starter

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cultures but the smear strains contribute to lipolysis and proteolysis (Rattray, 1996).

Milk contains a range of fatty acids esterified to form triglycerides. When these are hydrolysed, the free fatty acids can be acted upon to produce further flavour and aroma compounds. The exact role of esterases in cheese ripening is not well understood but it has been suggested that they could play a part in the production of short chain free fatty acids which contribute to the flavour of cheese (Bhowmik and Marth, 1989; Khalid et al., 1990). Indigenous milk lipoprotein lipase is partially inactivated by pasteurisation and, in good quality milk, heat resistant lipases from pyschrotrophic microorganisms should be absent. For internally ripened cheeses such as Cheddar, starters and secondary starters represent the main source of esterases and lipases during ripening (Gobbetti et al., 1997); however, in smear cheeses the degree of lipolysis is determined mainly by the enzymes of the surface microflora (Rattray, 1996).

Proteolysis in cheese can be seen as a cascade of reactions starting with proteins and leading to the production of free amino acids which are then metabolised. Casein is hydrolysed initially by enzymes (e.g., chymosin) from the rennet preparation used to coagulate milk, and plasmin, the principal indigenous proteinase present in the cheese milk. Coagulant and enzymes of the starter and non-starter flora of the cheese act on the products of these reactions (large and intermediate sized peptides) leading to the production of small peptides and free amino acids (Fox and McSweeney, 1996).

It is known that there is not necessarily a correlation between cheese flavour and concentration of free amino acids (Engels and Visser, 1994). However, these amino acids can be metabolised by bacterial enzymes to further products, including flavour compounds or their precursors (Hemme et al., 1982; Weimer et al., 1999). For example, Jollivet et al. (1992) reported that *Brevibacterium linens* produces ammonia, amines, acids, alcohols, aldehydes, 3-methylbutyric acid and caproic acid as a result of amino acid catabolism. The catabolism of sulphur amino acids plays a particularly important role in flavour. For example, L-methionine can be degraded to volatile compounds including methanethiol and *S*-methylthioesthers (Weimer et al., 1999).

The secondary flora of cheese catabolise free amino acids using enzymes such as transaminases, deaminases and carboxylases. Transaminases, or aminotransferases, catalyse the transfer of the amino group from an α-amino acid to an α-keto acid. These enzymes have broad substrate specificity (Weimer et al., 1999). Cystathionine lyases are pyridoxal-5′-phosphate (PLP)-dependent enzymes which metabolise cystathionine to cysteine, α-ketobutyrate and ammonia (α, γ elimination reaction), or to homocysteine, pyruvate and ammonia (α, β reaction) (Weimer et al., 1999).

An understanding of the enzymatic systems of smear bacteria could help in elucidating the role of these bacteria in ripening and indeed lead to a better understanding of cheese ripening itself. In this study, nine bacteria from two smear-ripened cheeses (Tilsit and Gubeen) were screened. The bacteria were assayed for aminopeptidase, di- and tri-peptidase, esterase, cystathionine lyase and L-methionine aminotransferase activities.

2. Materials and methods

2.1. Cultivation of bacteria

Corynebacterium casei B and *Corynebacterium* spp. I were from the culture collection of Teagasc, Moorepark, Fermoy, Ireland and were isolated from Gubeen, an Irish farmhouse cheese. Strains isolated from Tilsit cheese included *C. ammoniagenes* CA8, *Staphylococcus equorum* 6 and 14, *B. linens* 9 and 10 and *Brachybacterium* spp. 1 and 16, and were from the culture collection of the Institute of Microbiology, Federal Dairy Research Institute, Kiel, Germany. The strains were cultivated in a medium consisting of casein hydrolysate (10 g/l, BDH, Poole, England), peptone (5 g/l, Acumedia Manufacturers, Baltimore, MD), yeast extract (2.5 g/l, Acumedia Manufacturers), glucose (1 g/l, Sigma-Aldrich Chemie, Steinheim, Germany), skim milk powder (1 g/l, Difco Laboratories, Detroit, MI), and NaCl (30 g/l), and stored in 30% glycerol at −20 °C. The strains were incubated at 30 °C with agitation.

2.2. Cellular fractionation

Cells were lysed by one of two methods. For the proteolytic and esterolytic assays, cells were lysed using the method of Rattray and Fox (1997). This involved incubating lysozyme (Sigma-Aldrich) with

the pellet produced after centrifugation (Sorvall RC-5B Refrigerated Superspeed Centrifuge, DuPont Instruments, UK) of broth, washing of cells and recentrifugation. DNase and RNase (Boehringer Mannheim, Mannheim, Germany) were added and the cell lysate was recentrifuged. The resulting supernatant was termed the cell extract (CE).

For the screening for amino acid catabolic enzymes, cellular extracts [cell free supernatant, cell wall (CW) and cytoplasmic extract (CYT)] were obtained by the method of Coolbear et al. (1992) with several modifications. The extraction buffers contained 1 mM ethylenediaminetetracetic acid (EDTA, BDH) and 0.01 mM pyridoxal-5′-phosphate (PLP, Sigma-Aldrich). After centrifugation (11 000 × *g*, 10 min, 4 °C) of 1 l of bacterial cultures, grown in the broth described above, the supernatant was recovered as cell free supernatant. The cells were washed with 50 mM Tris–HCl, pH 7.5 containing 0.1 M $CaCl_2$ (Riedel-deHaen, Seelze, Germany), and recentrifuged. Following resuspension in Tris–HCl (50 mM, pH 7.5), and incubation at 30 °C for 30 min, the sample was centrifuged at 14 000 × *g* for 10 min at 4 °C. The pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.5, containing 24% (w/v) sucrose (Standard Chemicals, Waterford, Ireland) and $MgCl_2$ (10 mM, Merck-Darmstadt, Darmstadt, Germany). After incubation at 37 °C for 30 min, lysozyme was added (10 mg/ml). Following incubation at 37 °C with shaking (Lab Shaker, Adolf Kohner, Switzerland) at 150 rpm for 1.5 h, the samples were centrifuged (21 000 × *g*, 10 min, 20 °C). The supernatant was recovered as the CW fraction. The precipitate was resuspended in Tris–HCl, 20 mM, pH 7.5 and ultrasonically treated for 10 cycles of 30 s (Ultrasonic A180G Instrument, PBI International, Milan, Italy). After the addition of 40 ml of more buffer, the sample was incubated at 30 °C for 30 min then centrifuged at 25 000 × *g* for 30 min. The supernatant was recovered as cytoplasmic extract. The fractions were then dialysed against 50 times their volume of buffer (Tris–HCl, 20 mM, pH 7.5) at 4 °C for 24 h and then frozen at −20 °C until analysed.

2.3. Enzyme assays

2.3.1. Aminopeptidase

Aliquots of CE (50 μl) were incubated with substrate (Leu-pNA or His-pNA, 10 mM, Sigma-Aldrich), in reaction buffer (Tris–HCl, 50 mM, pH 7.5), for 1 h at 30 °C. The reaction was stopped by the addition of acetic acid (30%) and absorbance was determined at 410 nm (Cary 1E UV–VIS Spectrophotometer, Varian Australia, Australia).

2.3.2. Di- and tri-peptidase activity

Cd–Ninhydrin (cadmium chloride was from Merck, ninhydrin from Sigma-Aldrich) was used to study activity of the CE enzymes on five dipeptides (His–Leu; Ala–Met; Met–Ala; Pro–Leu; Pro–Ala, all from Sigma-Aldrich) and three tripeptides (Leu–Ala–Pro; Gly–Gly–Gly; Phe–Gly–Gly, from Sigma-Aldrich). The reaction mixture consisted of 50 μl CE, 50 μl substrate (1 mM in methanol) and 400 μl Tris–HCl (50 mM, pH 7.5), and was incubated at 40 °C for 10 min (Folkertsma and Fox, 1992). Cd–Ninhydrin (1 ml) was added, mixed and heated at 84 °C for 5 min. The samples were then placed in ice before absorbance was determined at 507 nm.

2.3.3. Esterases

The method of Gobbetti et al. (1997) was used to determine esterase activity. Substrates used were β-naphthyl derivatives of three fatty acids—butyrate, caprate and palmitate (Sigma-Aldrich). These were incubated with 20 μl CE in 200 μl Tris–HCl (50 mM, pH 7.5) for 2 h at 30 °C. Colour was developed by the addition of Fast Garnet GBC (5 mg/ml in 10% SDS, Sigma-Aldrich) followed by further incubation at room temperature for 15 min. Absorbance was determined at 560 nm.

To account for varying growth rates of the different strains, all esterolytic and proteolytic activities were adjusted for OD_{600}.

2.3.4. L-Methionine aminotransferase

The rate of formation of L-glutamic acid was used to assay for aminotransferase activity. The assay was based on the method of Yvon et al. (1997) with some modifications. The mixture contained 300 μl L-methionine (10 mM, Carlo Erba Reagenti, Milan, Italy), 50 μl PLP (1 mM), 50 μl α-ketoglutarate (200 mM, Sigma-Aldrich) and Tris–HCl (0.2 M, pH 8.0), in a final volume of 0.75 ml. This mixture was preincubated at 37 °C for 5 min. CYT (200 μl) was added and the mixture incubated for a further

15 min. After the addition of 50 μl sulfosalicyclic acid (60%, Hopkins and Williams, Essex, England), the assay mixture was left at 0 °C for 10 min and centrifuged (17 000 × *g*, 5 min; MSE Micro-Centaur, Sanyo Gallenkemp, Leicester, UK). The glutamate produced during the reaction was determined by colorimetric assay using an enzyme assay kit (Boehringer Mannheim). Controls without cytoplasmic extract and without substrate were included. One unit of activity (*c*) was expressed as the concentration of glutamic acid produced (g l^{-1} sample solution). Specific activity (U mg^{-1}) = *c* per mg of protein.

2.3.5. Cystathionine lyases

The cystathionine lyase activities were assayed by measuring the amount of free thiols formed from cystathionine (Ferchichi et al., 1985). The method is based on the principle that 5, 5′-dithiobis(2-nitrobenzoic acid, DTNB) (Sigma-Aldrich) reacts with the free thiols producing a colour which can be detected at 412 nm. The reaction mixture, consisting of 2 μl DTNB (5 mM in ethanol), 25 μl cystathionine (15 mM, Fluka Biochemica, Buchs, Switzerland), 2.5 μl sodium azide (final concentration of 0.05%, Sigma-Aldrich), 320 μl cell free supernatant, CW, or CYT and 16 μl reaction buffer (Tris–HCl, pH 8.0), was incubated at 30 °C. Controls without substrate and without enzyme sample were included. After a suitable incubation time, the reaction mixture was centrifuged at 25 000 × *g* for 2 min, and absorbance was read. One unit of activity was defined as an increase in absorbance of 0.01 in 10 min. Specific activity was expressed as number of units (U) per mg of protein.

This method cannot distinguish between cystathionine-γ-lyase (CγL) and cystathionine-β-lyase (CβL) activities. The presence of cysteine, as detected by ninhydrin method (Gaitonde, 1967), was used to differentiate between the enzyme activities. The ninhydrin reagent (consisting of 250 mg ninhydrin in 6 ml of acetic acid and 4 ml concentrated HCl) reacts with the cysteine in the sample, forming a pink colour, which is detectable at 560 nm. The presence of a pink colour was taken to indicate cystathionine-γ-lyase activity since cysteine is one of the products produced in CγL-catalysed reactions (Yamagata et al., 1993).

2.4. Protein determination

Protein concentration was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (Sigma-Aldrich) as the standard protein.

2.5. Statistical analysis

Results were analysed by Minitab Release 11.21 for Windows (Minitab, State College, PA). One-way analysis of variance was performed on the log data. In cases where $P < 0.05$, Tukeys Pairwise Comparisons (family error rate of 0.05) was performed.

3. Results

3.1. Proteolytic and esterolytic activities

3.1.1. Aminopeptidases

Table 1 shows activity of the cell extracts on Leu-pNA and His-pNA. Most strains showed more activity on Leu-pNA. However, *C. casei* B had equal activity

Table 1
Aminopeptidase activity ($\times 10^3$) of cell extracts on Leu-pNA and His-pNA

Strain	Activity on Leu-pNA	Activity on His-pNA	*P*
CA8	7.13 ± 5^{a}	6.03 ± 5^{a}	NS
CCB	1.14 ± 0.3^{b}	1.14 ± 0.3^{bd}	NS
CI	0.19 ± 0.2^{c}	ND	ND
SE6	0.44 ± 0.04^{Abc}	0.36 ± 0.02^{Bbe}	*
SE14	6.45 ± 0.9^{a}	7.62 ± 2^{ad}	NS
BY1	1.04 ± 0.3^{b}	0.68 ± 0.3^{b}	NS
BY16	0.21 ± 0.03^{Ac}	0.15 ± 0.01^{Bce}	*
BL9	0.94 ± 0.2^{b}	0.76 ± 0.3^{b}	NS
BL10	1.46 ± 0.2^{Ab}	0.168 ± 0.02^{Bce}	***
P	***	***	***

One unit of activity is expressed as absorbance at 412 nm adjusted for optical density at 600 nm.

Strains: CA8 — *C. ammoniagenes* CA8; CCB — *C. casei* B; CI — *Corynebacterium* spp. I; SE6 — *S. equorum* 6; SE14 — *S. equorum* 14; BY1 — *Brachybacterium* spp. 1; BY16 — *Brachybacterium* spp. 16; BL9 — *B. linens* 9; BL10 — *B. linens* 10.

NS: not significant; ND: not determined.

Values in the same column followed by th e same lowercase letter are not significantly different ($P > 0.05$). Values in the same row followed by the same capital letter are not significantly different.

* $P < 0.05$.

*** $P < 0.001$.

on both substrates. While *Corynebacterium* spp. I had no activity on His-pNA, *S. equorum* 14 displayed greater activity on this aminopeptidase substrate (7.7×10^{-3} U) than on Leu-pNA (7×10^{-3} U). Statistical analysis showed that there were significant differences ($P < 0.05$) between activities on Leu-pNA and His-pNA for *S. equorum* 6, *Brachybacterium* spp.16 and *B. linens* 10. *C. ammoniagenes* CA8 and *S. equorum* 14 had the greatest activity on Leu-pNA and His-pNA with activities of 7.2×10^{-3} and 7.5×10^{-3} U, respectively. *S. equorum* 6 and both brachybacteria had low activity on both substrates.

Strains with significantly different activities on either Leu- or His-pNA are shown in Table 1. On both substrates, mean values for activities of *C. ammoniagenes* CA8 were significantly different ($P < 0.05$) to all other strains except *S. equorum* 14.

3.1.2. Dipeptidases and tripeptidases

The brevibacteria showed greater activity on the dipeptidase rather than the tripeptidase substrates tested (Tables 2 and 3). This was also true of the brachybacteria. For *C. casei* B and *B. linens* 9, activity on Met–Ala was high compared to activity on the other peptides. There were no statistically significant differences between activity of *B. linens* 9 on Met–Ala and Ala–Met, or on Met–Ala and Pro–Leu. For *C. casei* B, there were significant differences between Pro–Ala and all of the other dipeptidase substrates. This was also true for *Brachybacterium* spp. 1. However, for *Corynebacterium* spp. I and *B. linens* 10, there were no significant differences between activities on any of the substrates. *C. casei* B had good activity on all five dipeptides. For both *C. ammoniagenes* CA8 and *Brachybacterium* spp. 1, Pro–Ala was the preferred dipeptide substrate. While *S. equorum* 6 had very low activity on all the substrates assayed, it was similar to *S. equorum* 14 in having greatest activity on Ala–Met (2.67×10^{-3} U for *S. equorum* 6 and 97.02×10^{-3} U for *S. equorum* 14) and Pro–Leu (2.54×10^{-3} U for *S. equorum* 6 and 94.68×10^{-3} U for *S. equorum* 14). On all substrates except Ala–Met, *S. equorum* 6 had significantly different activity to all other strains. On Pro–Ala only *C. ammoniagenes* CA8 and *Brachybacterium* spp. 16, and *C. casei* B and *B. linens* 9 had no significant differences.

C. casei B had good activity on the three tripeptidase substrates; however, the strain with highest activity on all three substrates was *S. equorum* 14 (Table 2). All strains, with the exception of the corynebacteria, had lowest activity on Leu–Ala–Pro. While *C. casei* B and *Corynebacterium* spp. I had activity on Gly–Gly–Gly, uniquely, *C. ammoniagenes* CA8 had no activity on this tripeptide. In contrast, both brachybac-

Table 2
Activity ($\times 10^3$) of smear strains on five dipeptidase substrates

Strain[b]	Met–Ala	Ala–Met	Pro–Leu	His–Leu	Pro–Ala	*P*
CA8	2.25 ± 1^{Aa}	8.23 ± 5^{Ba}	8.77 ± 0.5^{Ba}	20.30 ± 0.5^{Ba}	32.91 ± 5^{Ca}	***
CCB	84.10 ± 10^{Ab}	80.40 ± 10^{Ab}	75.354 ± 7^{Ab}	75.85 ± 6^{Ab}	53.32 ± 7^{Bb}	**
CI	ND	18.11 ± 2^{ce}	29.81 ± 20^{c}	60.74 ± 40^{be}	12.26 ± 0.6^{c}	NS
SE6	0.67 ± 0.2^{Ac}	2.67 ± 0.4^{Bd}	0.95 ± 0.01^{Ad}	0.71 ± 0.08^{Ac}	2.52 ± 0.4^{Bd}	***
SE14	27.85 ± 3^{Ad}	97.02 ± 20^{Bb}	30.78 ± 2^{Ac}	57.45 ± 6^{Cbe}	94.68 ± 0.6^{Be}	***
BY1	3.53 ± 0.6^{Aa}	2.62 ± 0.6^{Ad}	3.41 ± 0.3^{Ae}	2.57 ± 0.6^{Ad}	17.74 ± 2^{Bf}	***
BY16	8.98 ± 3^{Ae}	28.74 ± 2^{Bce}	8.71 ± 1^{Aa}	19.07 ± 0.9^{Ba}	29.09 ± 3^{Ba}	***
BL9	65.33 ± 0.7^{Abe}	54.83 ± 8^{ACbe}	55.70 ± 2^{ACbc}	36.66 ± 3^{Bab}	49.82 ± 3^{Cb}	***
BL10	33.11 ± 2^{de}	36.88 ± 0.6^{bc}	38.84 ± 6^{bc}	29.02 ± 4^{ae}	ND	NS
P	***	***	***	***	***	

Activity is absorbance at 507 nm adjusted for optical density.

Strains: CA8 — *C. ammoniagenes* CA8; CCB — *C. casei* B; CI — *Corynebacterium* spp. I; SE6 — *S. equorum* 6; SE14 — *S. equorum* 14; BY1 — *Brachybacterium* spp. 1; BY16 — *Brachybacterium* spp. 16; BL9 — *B. linens* 9; BL10 — *B. linens* 10.

ND: not determined; NS: not significant.

Values in the same column followed by the same lowercase letter are not significantly different ($P > 0.05$). Values in the same row followed by the same capital letter are not significantly different.

** $P < 0.01$.

*** $P < 0.001$.

Table 3
Tripeptidase activity ($\times 10^2$) of cell extracts on three substrates

Strain	Activity on Leu–Ala–Pro	Activity on Phe–Gly–Gly	Activity Gly–Gly–Gly	*P*
CA8	1.48 ± 0.4^{a}	1.26 ± 0.1^{a}	ND	NS
CCB	6.23 ± 1^{b}	6.40 ± 0.5^{b}	5.72 ± 0.6^{ac}	NS
CI	1.74 ± 0.9^{a}	2.55 ± 0.2^{c}	0.94 ± 0.9^{be}	NS
SE6	0.23 ± 0.01^{c}	0.27 ± 0.04^{d}	0.33 ± 0.08^{b}	NS
SE14	6.98 ± 2^{Ab}	8.38 ± 0.9^{ABe}	12.19 ± 1^{Ba}	*
BY1	0.57 ± 0.06^{Acd}	0.66 ± 0.03^{Af}	0.98 ± 0.1^{Bbf}	**
BY16	1.88 ± 0.3^{Aa}	2.27 ± 0.2^{Ac}	2.92 ± 0.09^{Bcf}	**
BL9	1.80 ± 0.3^{a}	2.19 ± 0.08^{c}	2.19 ± 0.4^{cf}	NS
BL10	0.94 ± 0.4^{ad}	1.40 ± 0.06^{a}	1.33 ± 0.2^{def}	NS
P	***	***	***	

One unit of activity is expressed as absorbance at 412 nm adjusted for optical density.
Strains: CA8 — *C. ammoniagenes* CA8; CCB — *C. casei* B; CI — *Corynebacterium* spp. I; SE6 — *S. equorum* 6; SE14 — *S. equorum* 14; BY1 — *Brachybacterium* spp. 1; BY16 — *Brachybacterium* spp. 16; BL9 — *B. linens* 9; BL10 — *B. linens* 10.
ND: not determined; NS: not significant.
Values in the same column followed by the same lowercase letter are not significantly different ($P > 0.05$). Values in the same row followed by the same capital letter are not significantly different.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

teria and both staphylococci had higher activity on Gly–Gly–Gly than on the other two substrates.

There were no significant differences ($P < 0.05$) between activity on Leu–Ala–Pro and Phe–Gly–Gly for any of the strains. There were differences observed between activity on Leu–Ala–Pro and Gly–Gly–Gly, and Phe–Gly–Gly and Gly–Gly–Gly for *B. linens* 9, *Brachybacterium* spp. 16 and *Brachybacterium* spp. 1. Comparing the activities of strains on individual substrates, it was found that there were generally significant differences between strains. For example, on Leu–Ala–Pro, *S. equorum* 6 was significantly different to all other strains apart from *Brachybacterium* spp. 1.

When activities of strains on Phe–Gly–Gly were compared, it was noted that there were significant differences in activity between the majority of strains. There were no differences between the activities of *Corynebacterium* spp. I and *Brachybacterium* spp. 16, *Corynebacterium* spp. I and *B. linens* 9, *C. ammoniagenes* CA8 and *B. linens* 10, *Brachybacterium* spp. 16 and *B. linens* 9.

3.1.3. Esterases

Table 4 shows the activity of the strains on the three esterase substrates. *C. ammoniagenes* CA8 and *S. equorum* 14 were most active on the three esterified fatty acids studied. *S. equorum* 6 and the two brachybacteria had low activity on all substrates. There was no preferred esterase substrate within the groups of related bacteria. For example, the greatest activity was observed on β-naphthyl caprate ($C_{10:0}$) for *C. ammoniagenes* CA8 (2.094 U), whereas *C. casei* B had greater activity on β-naphthyl butyrate ($C_{4:0}$) with an activity of *ca.* 0.24 U.

For the majority of strains, there were no significant differences between activities on the three esterase substrates. Activity of *Brachybacterium* spp. 1 was significantly different on β-naphthyl butyrate and β-naphthyl caprate. The other brachybacterium, *Brachybacterium* spp. 16, showed differences between activities on β-naphthyl butyrate and β-naphthyl palmitate, as well as between β-naphthyl caprate and β-naphthyl palmitate.

When activities of strains were compared on individual substrates, many significant differences were observed. However, all three substrates, there were no differences between the activities of *Corynebacterium* spp. B and *B. linens* 10 and *Brachybacterium* spp. 1 and *Brachybacterium* spp. 16.

3.2. Amino acid catabolic enzymes

The bacterial cell fractions (CW, supernatant and CYT) were screened for two amino acid catabolic enzymes—cystathionine lyase (CL) and L-methionine

Table 4
Esterase activity of cell extracts on three substrates

Strain	Activity on β-naphthyl butyrate	Activity on β-naphthyl caprate	Activity on β-naphthyl palmitate	*P*
CA8	1.85 ± 0.05^{a}	2.07 ± 0.05^{a}	2.04 ± 0.2^{a}	NS
CCB	0.26 ± 0.03^{b}	0.23 ± 0.01^{b}	0.25 ± 0.006^{b}	NS
CI	0.68 ± 0.003^{c}	0.65 ± 0.02^{c}	0.65 ± 0.06^{c}	NS
SE6	0.07 ± 0.008^{d}	0.08 ± 0.001^{d}	0.07 ± 0.006^{d}	NS
SE14	1.06 ± 0.03^{e}	1.12 ± 0.02^{e}	1.02 ± 0.6^{e}	NS
BY1	0.1 ± 0.003^{Afh}	0.113 ± 0.006^{Bf}	0.11 ± 0.006^{ABfh}	*
BY16	0.11 ± 0.006^{Agh}	0.117 ± 0.006^{Af}	0.09 ± 0.009^{Bgh}	*
BL9	0.31 ± 0.02^{b}	0.304 ± 0.02^{g}	0.31 ± 0.02^{b}	NS
BL10	0.23 ± 0.02^{b}	0.237 ± 0.02^{b}	0.22 ± 0.02^{b}	NS
P	***	***	***	

One unit of activity is expressed as absorbance at 412 nm adjusted for optical density.
Strains: CA8 — *C. ammoniagenes* CA8; CCB — *C. casei* B; CI — *Corynebacterium* spp. I; SE6 — *S. equorum* 6; SE14 — *S. equorum* 14; BY1 — *Brachybacterium* spp. 1; BY16 — *Brachybacterium* spp. 16; BL9 — *B. linens* 9; BL10 — *B. linens* 10.
ND: not determined; NS: not significant.
Values in the same column followed by the same lowercase letter are not significantly different ($P > 0.05$). Values in the same row followed by the same capital letter are not significantly different.
* $P < 0.05$.
*** $P < 0.001$.

aminotransferase (L-Met AT). Activity was detected only in the CYT, so this fraction was used subsequently for all enzyme assays (results not shown).

Aminotransferase activity was only detected in one strain — *S. equorum* 14. The enzyme had a specific activity of 22.8 U mg^{-1}.

All strains had activity on L-cystathionine (Table 5). *C. ammoniagenes* CA8 had the greatest specific activity (16.6 U/mg) with both brevibacteria also having relatively high activity. *Brachybacterium* spp. 1 was the least active strain (specific activity of 5.3 U mg^{-1}). All enzymes gave negative ninhydrin results. The activities of most strains were significantly different ($F = 838.46$, $P = 0.000$). However, there was no significant difference between the cystathionine lyase activities of *S. equorum* 6 and *Brachybacterium* spp. 16.

Table 5
Cystathionine lyase activity of smear strains

Strain	Specific activity U mg^{-1}
C. ammoniagenes 8	16.62 ± 1^{a}
C. casei B	9.34 ± 0.1^{b}
Corynebacterium spp. I	5.98 ± 0.02^{c}
S. equorum 6	7.69 ± 0.2^{d}
S. equorum 14	10.65 ± 0.2^{e}
Brachybacterium spp. 1	5.29 ± 0.3^{f}
Brachybacterium spp. 16	7.88 ± 0.03^{d}
B. linens 9	15.21 ± 0.5^{g}
B. linens 10	13.75 ± 0.2^{h}
P	***

One unit of activity is an increase in absorbance of 0.01 in 10 min.
NS: not significant; ND: not determined.
Values followed by the same lowercase letter are not significantly different ($P > 0.05$).
*** $P < 0.001$.

4. Discussion

Although the bacteria found on the surface of smear-ripened cheese are believed to contribute to ripening and flavour development in these cheeses, there has been little study on the enzymes from these strains, apart from the proteolytic system of brevibacteria (see Rattray and Fox, 1999). In an effort to understand the distribution of enzymatic activities among the strains and to compare these activities, nine smear strains were screened for activity on several peptides, fatty acids, cystathionine and methionine.

It must be taken into consideration that crude CEs were assayed for peptidase and esterase activities in this study. Therefore, activities on any substrate could be due to more than one enzyme or conversely activity on different substrates could be due to a single enzyme.

The peptidase system of lactic acid bacteria has been researched extensively; activities have been characterised and identified, e.g., PepN, PepC, PepT (Christensen et al., 1999). Similar research has not been done on the peptidases of smear strains.

Seven strains showed more activity on the aminopeptidase substrate containing a hydrophobic amino acid (Leu-pNA) than on His-pNA (basic amino acid). However, *S. equorum* 14 showed greater activity on His-pNA; this preference for basic rather than hydrophobic amino acids is similar to the PepN activity of LAB (Christensen et al., 1999). Although from the results obtained here (Table 1), it appears that many strains preferentially hydrolysed one of the two aminopeptides, in fact, statistically, only three strains had significantly different activity on the two substrates. Again, it should be remembered that the activities detected here could be as a result of one enzyme capable of hydrolysing both substrates, e.g., PepC.

Dipeptidase activity was widespread. Activity could not be determined for *Corynebacterium* spp. I on Met–Ala and *B. linens* 10 on Pro–Ala. With a small sample size, it was difficult to generalise, and it is not suggested that these results are true for all coryneform bacteria. However, it can be said for the strains assayed here, that the three corynebacteria had greatest activity on three different dipeptides. Additionally, both staphylococci preferred Ala–Met, while Pro–Ala was the favoured substrate for the two brachybacteria.

All strains, apart from *B. linens* 10, had some activity on Pro–Leu and on Pro–Ala. This is unsurprising since peptidases capable of hydrolysing Pro-containing sequences are important for cheese-related bacteria because of the presence of peptides from casein which have a high content of proline (Smacchi et al., 1999). Hydrolysis of proline-containing peptides increases the pool of amino acids available for bacterial growth.

It is also significant that all strains had activity on dipeptides containing a Met residue. If these enzymes were active during cheese ripening, their production of free methionine would facilitate amino acid catabolic activity on this sulphur-containing amino acid, leading to flavour compounds.

All the strains tested had activity on the tripeptide substrates, apart from *C. ammoniagenes* CA8 which was not active on Gly–Gly–Gly. Both *B. linens* 9 and *B. linens* 10 had least activity on Leu–Ala–Pro, as did the brachybacteria and staphylococci. In contrast, *C. casei* B and *Corynebacterium* spp. I had lowest activity on Gly–Gly–Gly, while no activity could be detected on this substrate for *C. ammoniagenes* CA8.

All strains had esterase activity. Interestingly, apart from the brachybacteria, for individual strains, the chain length of the fatty acid did not appear to affect the activity with strains displaying similar activity on the three substrates. This has previously been reported by Gobbetti et al. (1997) for *Lactobacillus plantarum*, but is in contrast to the findings of Williams and Banks (1997), who noted that specific activities of enzyme preparations from non-starter lactobacilli were lower when the carbon chain length of the esterified fatty acid was increased.

It is well known that the degradation of sulphur amino acids has a vital role in the development of the garlic aroma of smear ripened cheese. Methanethiol, a metabolite of methionine, has been detected in Münster, Livarot and other smear cheeses (Reps, 1993), as well as being a typical component of Cheddar cheese flavour (Hemme et al., 1982; Weimer et al., 1999). In this study, two enzymes involved in the catabolism of sulphur amino acids were studied—cystathionine lyase and L-methionine aminotransferase.

Preliminary experiments determined that these amino acid catabolic activities were located in the CYT fraction. Therefore, it was concluded that for the enzymes and strains screened here, the activities were intracellular. Alting et al. (1995) observed that lysis of cells is necessary for optimum activity of a lactococcal CβL during ripening. Dias and Weimer (1998a) suggested that the cells of *B. linens* BL2 must remain metabolically active during cheese ripening for the methionine-γ-lyase to have an effect on cheese flavour. However, Gao et al. (1998) reported that both whole and lysed cells of lactococci are capable of methanethiol formation, but these two types of cells use different pathways. If cell lysis occurs, the enzyme activities may be reduced due to the hostile environment. However, sulphur compounds have very low perception threshold values, e.g., 2 ppb for methanethiol (Weimer et al., 1999), so even with reduced activity, these enzymes can play a role in flavour development.

Aminotransferases catalyse the first step in the catabolism of many amino acids (Weimer et al., 1999). In this study, the only strain with L-Met AT activity was

S. equorum 14. Previously, aminotransferase activity was detected in one of two brevibacteria screened (Dias and Weimer, 1998b) but the two strains of *B. linens* assayed did not have this activity.

All the smear strains screened had cystathionine lyase activity. The majority of the strains (seven of nine) had significantly different activities on cystathionine. Although the main function of this enzyme is the catabolism of cystathionine, it could be significant in flavour development as enzymes previously isolated have also been shown to produce methanethiol (Alting et al., 1995; Bruinenberg et al., 1997; Dobric et al., 2000). While the precise role of cystathionine lyases in cheese ripening is unknown, Weimer et al. (1999) suggested that the addition of these enzymes to the cheese matrix may have a positive effect on ripening. The enzymes gave negative ninhydrin results. Since the ninhydrin assay measures cysteine, the results suggest that the enzymes used the α,β elimination reaction producing homocysteine, ammonia and pyruvate and were thus CβL.

There is little doubt that enzyme activities have vital roles in ripening and flavour development in cheese. Further study will be necessary to understand better the role of these bacterial enzymes in ripening of smear cheese.

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