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Evidence of a relationship between autolysis of starter bacteria and lipolysis in Cheddar cheese during ripening

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Cell viability, autolysis and lipolysis were studied in Cheddar cheese made using *Lactococcus lactis* subsp. *cremoris* AM2 or *Lactococcus lactis* subsp. *cremoris* HP. Cheddar cheese was made in triplicate over a 3 month period and ripened for 238 days at 8 °C. Cell viability in cheese was lower for AM2 (a non-bitter strain) than for strain HP (a bitter strain). Autolysis, monitored by the level of the intracellular marker enzyme, lactate dehydrogenase (EC 1.1.1.27) in cheese 'juice' extracted by hydraulic pressure, was much greater in the cheese made using AM2 than that made with HP. Lipolysis was determined by the increase during ripening of individual free fatty acids (FFA) from butyric ($C_{4:0}$) to linolenic acid ($C_{18:3}$) measured using a high performance liquid chromatographic technique. Levels of individual FFA from butyric ($C_{4:0}$) to linolenic ($C_{18:3}$) acids increased significantly ($P<0{\cdot}05$) during ripening in cheeses made with either starter culture. Palmitic ($C_{16:0}$) and oleic ($C_{18:1}$) acids were the most abundant FFA throughout ripening in all cheeses. Levels of caprylic ($C_{8:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids were significantly higher ($P<0{\cdot}05$) in cheeses manufactured with *Lc. lactis* subsp. *cremoris* AM2 than in cheeses manufactured with *Lc. lactis* subsp. *cremoris* HP. Differences in levels of lipolysis between strains was not due to differences in the specific lipolytic or esterolytic activities in cell free extracts of the strains as measured by activity on triolein (lipase) and *p*-nitrophenylbutyrate (esterase) substrates. Therefore, evidence is provided for a relationship between the extent of starter cell autolysis and the level of lipolysis during Cheddar cheese ripening.

Keywords: Lipolysis, autolysis, lactate dehydrogenase, lipase, free fatty acids.

Lipolysis makes an important contribution to overall flavour development in cheese during ripening, especially in varieties such as Blue and hard Italian types and is mediated by lipases originating from milk, starter, non-starter and secondary starter bacteria (Anderson et al. 1991). While milk contains a very potent indigenous lipoprotein lipase (LPL), it normally never reaches its full activity in milk (Fox & Stepaniak, 1993; Fox et al. 1993) and high-temperature short-time (HTST) treatment (72 °C for 15 s), very extensively inactivates the enzyme (Deeth & Fitz-Gerald, 1983). However, it is still thought to contribute to lipolysis in pasteurized-milk cheese as a time and temperature combination of 78 °C for 10 s is required for its complete inactivation (Driessen, 1989). Lipases (triacylglycerol acylhydrolases; E.C. 3.1.1.3) are an ubiquitous group of enzymes that catalyse the hydrolysis of triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids and glycerol (Deeth & Fitz-Gerald, 1995; Thomson et al. 1999) having maximum activity at water–oil interfaces (Louwrier et al. 1996; Verger, 1997) and many also exhibit positional specificity (Marangoni & Rousseau, 1995; Ramamurthi & McCurdy, 1995) for the esters at the *sn*-1 and *sn*-3 positions of the triacylglycerol molecule (Olivecrona & Bengtsson-Olivecrona, 1991; Fox et al. 1996*a*). Lipase activity can be measured by hydrolysis of emulsified triacylglycerols of long chain fatty acids with the most common substrates being tributyrin and triolein (Peled & Krenz, 1981; Jensen, 1983; Papaparaskevas et al. 1992).

Lipolytic degradation of triglycerides of milk fat during cheese ripening results in the release of free fatty acids (FFA) which are further catabolized to highly flavoured compounds including methyl ketones, thioesters and lactones. FFA, particularly short chain fatty acids, are highly

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flavoured and, at high levels, butyric acid and other short chain fatty acids have been associated with the lipolysed flavour defect or rancidity in Cheddar cheese (Deeth & Touch, 2000).

Lipolysis in mould or smear ripened cheeses has been studied in most detail and has been shown to originate from the lipolytic action of enzymes from the mould or other secondary cultures (Anderson & Day, 1966; Dartey & Kinsella, 1973; King & Clegg, 1979; Gripon, 1993; Reps, 1993; Tomasini et al. 1993; Molimard & Spinnler, 1996). However, in the case of Cheddar cheese, information on the contribution of lipolysis to flavour development and whether or not it is influenced by the degree of starter autolysis is quite limited. Previously, it has been shown that starters with enhanced autolytic abilities can accelerate proteolysis and hence ripening of cheese through an early release of intracellular enzymes (Crow et al. 1993, 1995; Chapot-Chartier, 1994; Wilkinson et al. 1994*a*, *b*; O'Donovan et al. 1996). Lemee et al. (1994) proposed that autolysis of *Propionibacterium freudenreichii* could contribute to lipolysis in Swiss cheese, based on the earlier identification of intracellular esterase activities in *Prop. freudenreichii* by Dupuis et al. (1993), however lipolysis was not monitored in the former study. The effect of starter autolysis on lipolysis in cheese has received relatively little attention.

The objectives of this study were to quantify lipolysis during Cheddar cheese ripening and to establish whether a relationship exists between the degree of starter cell autolysis and levels of lipolysis in cheese.

Materials and Methods

Starter cultures for cheesemaking

Lactococcus lactis subsp. *cremoris* AM2 and *Lactococcus lactis* subsp. *cremoris* HP were obtained from the culture collection of the Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork. The cultures were maintained in reconstituted skim milk (RSM, 100 g/l) at −80 °C and transferred twice in RSM prior to cheesemaking.

Estimation of lipolytic and esterolytic activities of the cell free extracts (CFE) of starter cultures used for cheesemaking

The starters used for cheesemaking (AM2 and HP) were propagated at 30 °C for *ca.* 7 h in 450 ml RSM which had been centrifuged at 5000 ***g*** at 4 °C for 20 min and the supernatant buffered to pH 7·1 with 3 M β-glycerol phosphate (2·5 ml/100 ml). Propagation was terminated at pH 5·2 (exponential phase of growth). CFE were prepared in triplicate following the method of Bouchier (1999) with the following modifications; after washing the pellets, cells re-suspended in 8 ml Tris-HCl buffer (pH 7·5) were disrupted by beating for a total of 6 min in a bead beater (Biospec Products Inc., Bartlesville, OK 74005-0788, USA), using 0·1 mm zirconia/silica beads (Biospec Products Inc.). CFE was finally centrifuged at 14 000 ***g*** for 20 min to remove cell debris. Total nitrogen contents of CFE were estimated according to the Kjeldahl method (International Dairy Federation, 1986*a*).

Lipase activity of fresh CFE from the individual growth experiments was determined in triplicate using triolein emulsion as substrate according to the method of Tanaka et al. (1999). The following variations were used, triolein (2·5 ml) was emulsified with 7·5 ml polyvinyl alcohol solution (20 g/l) in a bench-top valve homogenizer type 134-930500 (Mecam, Sweden). Homogenization was carried out at 20 °C and at a pressure of 40 bar. Following the lipase reaction and addition of 5 ml chloroform-methanol (2 : 1, v/v) solution, which destroyed the emulsion, an aliquot (100 µl) was transferred to a new tube and heated at 50 °C for 20 min to evaporate the chloroform and methanol. Results were expressed as mmol oleic acid/l and 1 unit of lipase activity was defined as the amount of enzyme activity necessary to release 1 µEq oleic acid per min.

Esterolytic activity of fresh CFE from each growth experiment was measured in triplicate using the chromogenic substrate, *p*-nitrophenylbutyrate (PNPB) (Sigma-Aldrich Company Ltd., Dorset, BH12 4QH, UK). CFE (100 µl) was equilibrated with 900 µl phosphate buffer pH 7 (consisting of 100 mM-NaH_2PO_4–150 mM-NaCl and 5 ml Triton X-100/l) at 37 °C for 10 min. Substrate was then added (10 µl) and the assay mixture was incubated at 37 °C for 30 min. Hydrolysis of PNPB and concomitant release of the p-NP compound was indicated by an increase in absorbance at 400 nm. Samples were read against both sample and substrate blanks. Results were expressed as a change in absorbance per min and 1 unit of esterase activity was defined as the amount of enzyme necessary to release 1 nmol *p*-nitrophenol per min from PNPB at 37 °C and pH 7.

Cheddar cheese manufacture

Milk was separated at 63 °C, standardized to a protein : fat ratio of 0·9 : 1, pasteurized at 72 °C for 15 s and cooled to 31 °C. Cheese was manufactured in triplicate according to the method of Wilkinson et al. (1994*b*) and subsequently ripened at 8 °C for 238 d.

Extraction of cheese juice

Over a 238 d ripening period cheese juice and fat were extracted from grated cheese (300 g) mixed with sand (600 g) using hydraulic pressure (pressure was increased to 32 MPa over 1 h and expressed liquid was collected over the next 3 h at room temperature; Wilkinson et al. 1994*b*). Juice and fat were extracted in duplicate from cheese samples of three trials and frozen in 500 µl aliquots at −20 °C.

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Starter viability and lysis in cheese

Viability of starter lactococci in cheese was measured in duplicate, taking two independent samples from cheese from each of the three trials, on lactose M17 agar (Terzaghi & Sandine, 1975), incubated at 30 °C for 3 d, results were expressed as cfu/g cheese. Non-starter lactic acid bacteria (NSLAB) were enumerated in duplicate on *Lactobacillus*-selective agar (LBS) (Rogosa et al. 1951), incubated for 5 d at 30 °C under anaerobic conditions.

Autolysis was monitored by the release of lactate dehydrogenase (LDH, E.C. 1.1.1.27) in fresh cheese juice; activity was determined by the method of Wittenberger & Angelo (1970). Activity was expressed as units/ml press juice where 1 unit was defined as the amount of enzyme that catalysed the transformation of 1 µmol NADH/min per ml juice.

Cheese composition

Grated cheese samples obtained from each of the three cheese trials were analysed in duplicate for salt (International Dairy Federation, 1979), fat (International Dairy Federation, 1986*b*), total nitrogen (International Dairy Federation, 1986*a*) and moisture by drying to a constant weight at 102 °C (International Dairy Federation, 1982). pH of a paste, prepared by blending 12 ml H_2O at 40 °C with 20 g grated cheese, was measured using a pH meter (model 26, Radiometer, Copenhagen DK-2400, Denmark).

Monitoring of lipolysis in cheese

FFA from butyric ($C_{4:0}$) to linolenic ($C_{18:3}$) were quantified by resolution of *p*-bromophenacyl (PBP) derivatives of the FFA by RP-HPLC (Kilcawley et al. 2001). Samples of cheese from the three trials were assayed in duplicate at each sampling point. To enable better separation of fat, 0·2 ml of a 9·5 M solution of sulphuric acid was added to each 2·5 g sample of grated cheese. FFA were separated from the cheese by solvent extraction using diethyl ether and centrifuged, pellets were re-extracted twice as described by Kilcawley et al. (2001), however, the three individual solvent extracts were not pooled, but were treated as separate samples to facilitate easier identification of FFA peaks on the chromatogram. The quantified FFA in each extract were summed to give the total of individual FFA in the cheese sample. An internal standard, nonanoic acid ($C_{9:0}$; 0·1 mol/l (v/v)), was added to each solvent extract. Results were expressed as mg individual FFA/kg cheese.

Statistical analysis of FFA data

A split plot design with three replicates (trials) was used to analyse the effects of the three treatments, i.e., the starter strain, days of ripening, and the interaction between starter strain and days of ripening on the response variables (levels FFA). The main plot factor was starter strain and the sub-plot factor was days of ripening. The procedure used was a general linear model (GLM; Sas® Inc., Cary, NC 27513, USA) for analysis of variance of a split plot design. Fisher's least significant difference test was used to determine whether statistically significant differences had occurred among means. All differences considered as significant had P values $<0{\cdot}05$.

Table 1. Presence and specific activities of intracellular lipase, esterase and lactate dehydrogenase (LDH) in *Lactococcus lactis* subsp. *cremoris* AM2 and *Lc. lactis* subsp. *cremoris* HP grown in buffered reconstituted skim milk at 30 °C

Values are means ± SD for $n=3$

	AM2	HP
Lipase, µEq oleic acid released/min/mg protein	0·022 ± 0·009	0·071 ± 0·012
Esterase, abs/min/mg protein	0·013 ± 0·002	0·013 ± 0·003
LDH, units/mg protein	0·022 ± 0·032	2·984 ± 1·869

Results

Lipolytic and esterolytic activities of CFE of the starter cultures

Specific lipolytic, esterolytic and LDH activities of the CFE of the cultures *Lc. lactis* subsp. *cremoris* AM2 and *Lc. lactis* subsp. *cremoris* HP grown in RSM at 30 °C to pH 5·2 are presented in Table 1. Specific activity of LDH (units /mg protein) was highest in the CFE of *Lc. lactis* subsp. *cremoris* HP. Specific esterolytic activity was similar in CFE of *Lc. lactis* subsp. *cremoris* AM2 and *Lc. lactis* subsp. *cremoris* HP, while specific lipolytic activity was higher in the CFE of *Lc. lactis* subsp. *cremoris* HP.

Viability in cheese of starter and non-starter bacteria

Viabilities of the different starter strains in Trials 1, 2 and 3, detected on LM17 agar, are shown in Figs. 1a, b & c, respectively. Viable cell populations were generally higher for *Lc. lactis* subsp. *cremoris* HP on day 1 of ripening ($\sim 10^8$ cfu/g) compared with *Lc. lactis* subsp. *cremoris* AM2 ($\sim 10^6$ cfu/g) for all trials. However, over the first 8 weeks of ripening, viability of AM2 was lower than HP with populations of AM2 falling to $\sim 10^4$ cfu/g compared with $\sim 10^6$ cfu/g for strain HP.

In contrast to the decline in starter populations over the first 8 weeks of ripening, counts of NSLAB increased during ripening in all cheeses up to 238 d (Figs. 2a, b & c). Generally, NSLAB populations increased from $\sim 10^2$ cfu/g on day 1 to reach $\sim 10^7$ cfu/g at the end of the ripening period in cheeses from all three trials.

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Table 2. Composition of experimental Cheddar cheeses of Trials 1, 2 and 3 made with *Lactococcus lactis* subsp. *cremoris* AM2 or *Lc. lactis* subsp. *cremoris* HP as starter after 14 d of ripening

Values are means of duplicates

Starter	Trial	Fat (%)	Protein (%)	Moisture (%)	T.S.† (%)	pH	Salt (%)	S/M‡ (%)	F.D.M.§ (%)
AM2	1	30·797	25·56	38·09	61·91	5·22	2·01	5·277	49·745
	2	31·925	25·92	38·58	61·42	5·12	1·77	4·588	51·978
	3	30·546	25·28	38·21	61·79	5·21	1·93	5·051	49·435
HP	1	30·467	24·90	39·84	60·16	5·04	1·89	4·744	50·564
	2	31·613	25·47	38·75	61·25	5·07	1·91	4·929	51·613
	3	30·465	25·51	38·35	61·65	5·11	2·00	5·215	49·416

† Total solids
‡ Salt in moisture
§ Fat in dry matter

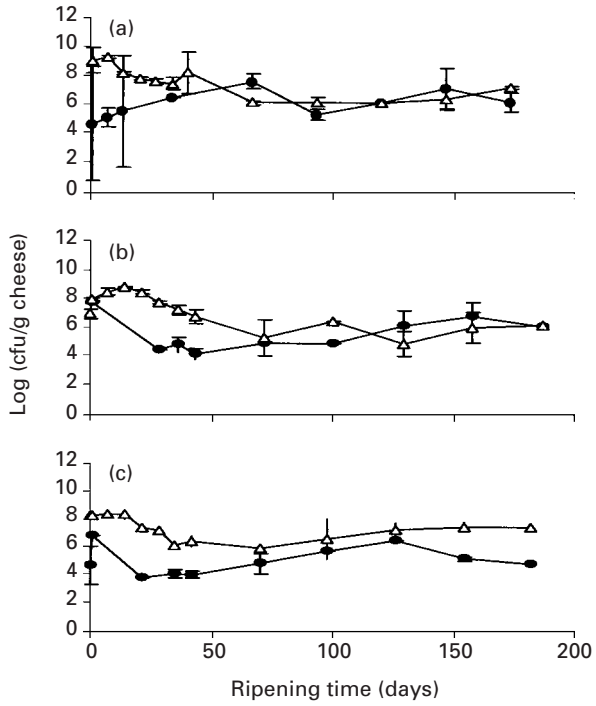

Fig. 1. Viable starter cell populations, expressed as log cfu/g cheese, enumerated on LM17 agar in Cheddar cheese manufactured with *Lc. lactis* subsp. *cremoris* AM2 (●) or *Lc. lactis* subsp. *cremoris* HP (△) as starter in Trials 1 (a), 2 (b) and 3 (c).

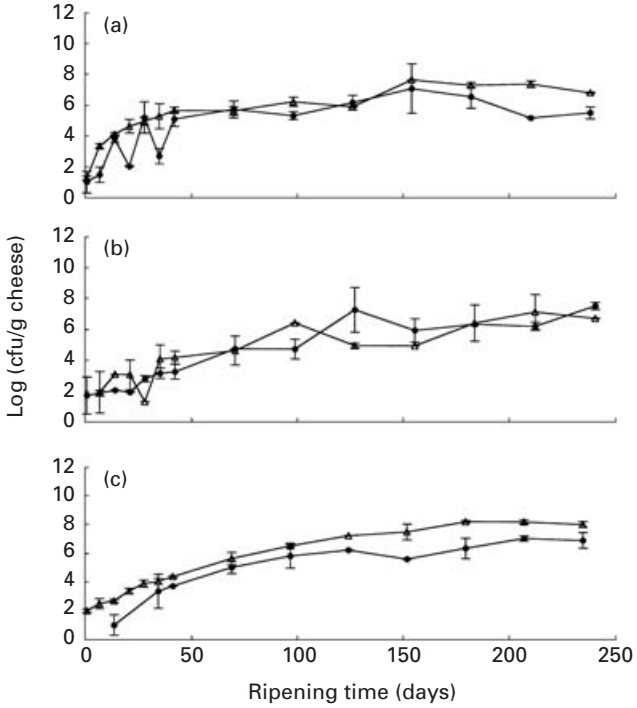

Fig. 2. Viable counts of Non-Starter Lactic Acid Bacteria (NSLAB), expressed as log cfu/g cheese, enumerated on LBS agar in Cheddar cheeses manufactured with *Lc. lactis* subsp. *cremoris* AM2 (●) or *Lc. lactis* subsp. *cremoris* HP (△) as starter in Trials 1 (a), 2 (b) and 3 (c).

Cell autolysis as measured by the release of the marker enzyme, LDH

LDH activity was detected in cheese juice extracted from both cheeses during ripening and results for trials 1, 2 and 3 are presented in Figs. 3a, b & c, respectively. At all sampling points during the 238 d ripening period in all three trials, activity of LDH in cheese juice was higher in cheese made with *Lc. lactis* subsp. *cremoris* AM2 than in cheese made with *Lc. lactis* subsp. *cremoris* HP.

Cheese composition

Compositional analyses at 14 d for cheeses made with *Lc. lactis* subsp. *cremoris* AM2 or *Lc. lactis* subsp. *cremoris* HP for the three trials are presented in Table 2. Compositions of cheese were similar in all three trials.

FFA levels in cheese during ripening

Levels of individual FFA from butyric ($C_{4:0}$) to linolenic ($C_{18:3}$) acids in cheeses made with *Lc. lactis* subsp. *cremoris*

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Table 3. Free fatty acid (FFA) levels, expressed as mg/kg cheese, in cheeses of Trial 1 manufactured with *Lactococcus lactis* subsp. *cremoris* AM2 or *Lc. lactis* subsp. *cremoris* HP as starter

Values are means and (SD) for $n=2$

	Day 1		Day 35		Day 70		Day 126		Day 182		Day 238	
FFA	AM2	HP	AM2	HP	AM2	HP	AM2	HP	AM2	HP	AM2	HP
$C_{4:0}$	151 (41)	70 (25)	28 (0)	30 (1)	101 (40)	73 (18)	40 (8)	32 (2)	114 (18)	58 (7)	93 (11)	54 (4)
$C_{6:0}$	59 (13)	87 (10)	46 (3)	44 (1)	79 (18)	108 (22)	55 (4)	54 (0)	65 (3)	57 (0)	80 (1)	79 (3)
$C_{8:0}$†	60 (17)	68 (7)	46 (12)	41 (2)	79 (4)	62 (14)	48 (4)	42 (3)	76 (7)	61 (0)	91 (17)	76 (15)
$C_{10:0}$	72 (1)	71 (1)	58 (10)	56 (8)	98 (2)	63 (12)	71 (12)	55 (7)	87 (7)	72 (9)	116 (18)	96 (22)
$C_{12:0}$	67 (3)	66 (2)	54 (5)	60 (3)	101 (1)	77 (6)	69 (6)	60 (5)	87 (3)	75 (4)	117 (19)	106 (20)
$C_{14:0}$†	127 (1)	138 (0)	109 (5)	112 (2)	181 (2)	159 (0)	135 (8)	119 (23)	158 (2)	144 (1)	200 (22)	181 (17)
$C_{16:0}$†	423 (25)	426 (3)	380 (76)	354 (48)	684 (1)	424 (82)	452 (79)	353 (53)	602 (32)	484 (64)	817 (148)	643 (114)
$C_{18:0}$†	214 (20)	207 (0)	188 (43)	165 (11)	287 (7)	208 (30)	189 (26)	158 (18)	255 (9)	216 (17)	330 (55)	280 (40)
$C_{18:1}$	726 (37)	695 (15)	580 (73)	567 (82)	1106 (38)	635 (123)	708 (135)	541 (80)	931 (64)	617 (64)	1313 (247)	1042 (211)
$C_{18:2}$	121 (5)	102 (4)	111 (32)	122 (2)	189 (3)	144 (1)	153 (18)	129 (12)	171 (32)	124 (16)	204 (39)	159 (40)
$C_{18:3}$	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	2020 (86)	1929 (18)	1599 (253)	1552 (156)	2904 (156)	1953 (257)	1919 (300)	1542 (203)	2546 (136)	1907 (53)	3362 (577)	2716 (479)

† Denotes FFA significantly higher ($P<0{\cdot}05$) in cheeses manufactured using *Lc. lactis* subsp. *cremoris* AM2 than in cheeses made using *Lc. lactis* subsp. *cremoris* HP

Table 4. Free fatty acid levels, expressed as mg/kg cheese, in cheeses of Trial 2 manufactured with *Lactococcus lactis* subsp. *cremoris* AM2 or *Lc. lactis* subsp. *cremoris* HP as starter

Values are means and (SD) for $n=2$

	Day 1		Day 35		Day 70		Day 126		Day 182		Day 238	
FFA	AM2	HP	AM2	HP	AM2	HP	AM2	HP	AM2	HP	AM2	HP
$C_{4:0}$	138 (13)	110 (1)	26 (4)	25 (1)	51 (13)	36 (40)	42 (10)	48 (10)	29 (1)	33 (2)	102 (11)	57 (7)
$C_{6:0}$	125 (11)	106 (10)	47 (9)	52 (3)	48 (2)	32 (1)	62 (7)	70 (7)	49 (1)	40 (4)	87 (7)	76 (14)
$C_{8:0}$†	68 (9)	70 (0)	37 (2)	39 (2)	47 (4)	26 (3)	54 (7)	64 (13)	49 (3)	33 (5)	88 (4)	73 (18)
$C_{10:0}$	53 (2)	64 (8)	42 (1)	45 (4)	56 (13)	33 (6)	62 (19)	74 (17)	61 (2)	43 (7)	110 (8)	85 (23)
$C_{12:0}$	60 (5)	113 (8)	50 (3)	45 (12)	54 (5)	37 (3)	64 (11)	71 (9)	52 (1)	44 (2)	99 (6)	74 (12)
$C_{14:0}$†	146 (6)	153 (6)	109 (9)	108 (2)	113 (1)	81 (6)	140 (14)	153 (11)	101 (1)	91 (3)	173 (8)	157 (18)
$C_{16:0}$†	368 (1)	463 (20)	298 (5)	306 (29)	371 (86)	229 (36)	428 (131)	535 (117)	387 (26)	303 (50)	741 (53)	579 (140)
$C_{18:0}$†	178 (8)	215 (24)	136 (7)	147 (8)	149 (19)	96 (9)	173 (34)	200 (23)	143 (7)	122 (18)	272 (17)	227 (47)
$C_{18:1}$	526 (8)	649 (21)	414 (21)	443 (43)	529 (75)	349 (63)	701 (235)	856 (204)	672 (29)	513 (76)	1316 (84)	979 (200)
$C_{18:2}$	130 (3)	137 (12)	100 (8)	99 (3)	110 (6)	75 (8)	128 (25)	147 (15)	108 (7)	92 (8)	180 (8)	160 (28)
$C_{18:3}$	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	1793 (50)	2079 (76)	1259 (69)	1309 (107)	1528 (196)	996 (132)	1855 (493)	2217 (426)	1653 (75)	1315 (175)	3168 (205)	2469 (506)

† Denotes FFA significantly higher ($P<0{\cdot}05$) in cheeses manufactured using *Lc. lactis* subsp. *cremoris* AM2 than in cheeses made using *Lc. lactis* subsp. *cremoris* HP

Table 5. Free fatty acid levels, expressed as mg/kg cheese, in cheeses of Trial 3 manufactured with *Lactococcus lactis* subsp. *cremoris* AM2 or *Lc. lactis* subsp. *cremoris* HP as starter

Values are means and (SD) for $n=2$

	Day 1		Day 35		Day 70		Day 126		Day 182		Day 238	
FFA	AM2	HP	AM2	HP	AM2	HP	AM2	HP	AM2	HP	AM2	HP
$C_{4:0}$	56 (13)	38 (0)	31 (5)	30 (0)	64 (0)	48 (11)	29 (1)	25 (0)	32 (1)	40 (14)	70 (8)	40 (10)
$C_{6:0}$	61 (8)	51 (1)	48 (3)	53 (2)	50 (5)	50 (0)	49 (1)	47 (1)	51 (5)	61 (1)	62 (0)	47 (8)
$C_{8:0}$†	44 (10)	44 (6)	33 (4)	35 (1)	35 (2)	33 (3)	36 (1)	31 (1)	37 (3)	36 (0)	49 (4)	44 (1)
$C_{10:0}$	52 (4)	52 (10)	41 (6)	37 (1)	38 (0)	31 (3)	46 (2)	36 (7)	44 (11)	47 (2)	52 (1)	49 (5)
$C_{12:0}$	49 (2)	50 (3)	47 (4)	42 (1)	70 (35)	42 (2)	52 (1)	44 (3)	50 (3)	52 (2)	117 (1)	88 (24)
$C_{14:0}$†	118 (12)	130 (3)	104 (2)	112 (3)	128 (0)	113 (10)	124 (3)	112 (3)	126 (3)	125 (2)	128 (0)	110 (25)
$C_{16:0}$†	317 (8)	295 (65)	228 (21)	214 (4)	236 (8)	201 (1)	271 (0)	210 (37)	260 (48)	278 (8)	368 (2)	282 (17)
$C_{18:0}$†	133 (3)	122 (12)	109 (14)	103 (0)	110 (4)	100 (1)	115 (1)	96 (7)	113 (8)	115 (2)	99 (3)	79 (22)
$C_{18:1}$	339 (18)	318 (83)	254 (24)	225 (5)	263 (16)	193 (3)	287 (1)	217 (46)	264 (51)	277 (14)	314 (1)	241 (74)
$C_{18:2}$	79 (7)	85 (1)	79 (1)	84 (1)	77 (1)	79 (5)	89 (3)	87 (2)	72 (22)	89 (1)	126 (2)	102 (17)
$C_{18:3}$	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	1249 (43)	1184 (181)	975 (84)	934 (15)	1072 (14)	888 (35)	1098 (8)	904 (107)	1048 (140)	1119 (47)	1385 (4)	1080 (281)

†Denotes FFA significantly higher ($P<0{\cdot}05$) in cheeses manufactured using *Lc. lactis* subsp. *cremoris* AM2 than in cheeses made using *Lc. lactis* subsp. *cremoris* HP

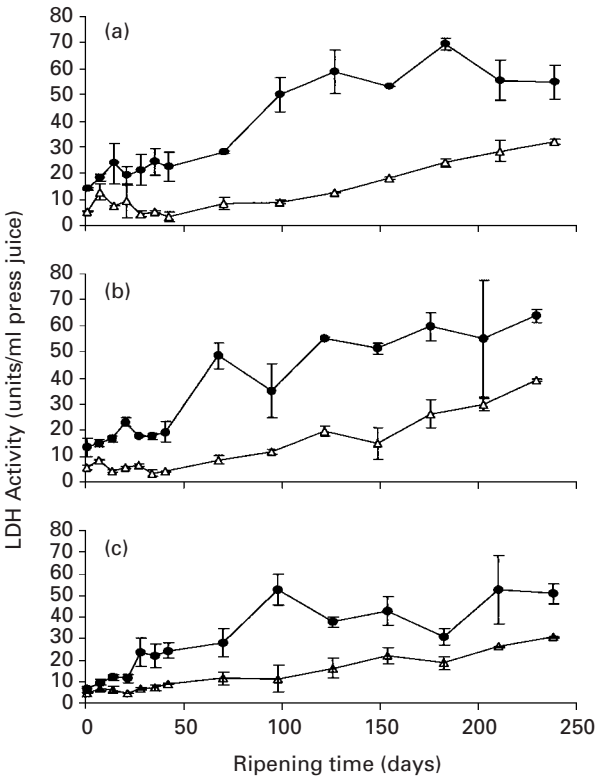

Fig. 3. LDH activity in cheese juice expressed from Cheddar cheeses manufactured with *Lc. lactis* subsp. *cremoris* AM2 (●) or *Lc. lactis* subsp. *cremoris* HP (△) as starter in Trials 1 (a), 2 (b) and 3 (c).

AM2 or *Lc. lactis* subsp. *cremoris* HP up to 238 d of ripening in trials 1, 2 and 3 are presented in Tables 3, 4 & 5, respectively. Throughout the ripening period, palmitic and oleic acids were the most abundant FFA in all cheeses and consequently the most pronounced increase over time was observed for these FFA. Levels of each FFA increased as ripening progressed up to 238 d in all cheeses, except for linolenic acid ($C_{18:3}$) which was not detected in any cheese and also butyric and caproic acids which did not show a considerable increase during ripening. Consistently lower levels of FFA during ripening were noted in the cheese from trial 3 compared with trials 1 and 2. Generally, over the three trials, as ripening progressed beyond day 70, the levels of individual FFA in the range $C_{4:0}$ to $C_{14:0}$ were somewhat higher in cheese made using *Lc. lactis* subsp. *cremoris* AM2 compared with those made with *Lc. lactis* subsp. *cremoris* HP. However, in the case of the longer chain FFA, palmitic ($C_{16:0}$) and oleic acid ($C_{18:1}$), from day 70 of ripening onwards, differences became quite large with highest levels found in cheeses made with *Lc. lactis* subsp. *cremoris* AM2.

Statistical analysis of FFA data

Data from 3 trials were analysed to determine whether statistically significant relationships existed between, starter

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strain used for cheese manufacture, days of ripening and the interaction between starter culture and days of ripening, on the release of FFA during ripening. Cheddar cheese manufactured using the highly autolytic strain *Lc. lactis* subsp. *cremoris* AM2 had significantly higher levels ($P<0{\cdot}05$) of caprylic ($C_{8:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids compared with the levels found in cheeses made with poorly autolytic strain *Lc. lactis* subsp. *cremoris* HP. However, while the overall effect of starter was significant, levels of certain FFA were found to be numerically lower for strain AM2 at some of the time points, particularly those in the earlier stages of ripening, see Tables 3–5. A similar significant effect ($P<0{\cdot}05$) on FFA release was found for days of ripening. In contrast, no effect was found for the interaction between starter culture and days of ripening.

Discussion

Lipolysis is an important biochemical event in the development of the final flavour of cheese and the ratios and levels of individual FFA are considered to influence the flavour of different cheese varieties (Woo et al. 1984), both directly, particularly in some Italian varieties, and indirectly, as precursors for other compounds such as methyl ketones, thioesters and lactones. While the exact nature of the contribution of FFA to the flavour of Cheddar cheese remains unclear, they have been suggested as being the backbone of Cheddar flavour (Patton, 1963) and have been reported to contribute to aroma of Cheddar flavour (Forss & Patton, 1966; Forss, 1979). It is generally agreed that FFA, at appropriate concentrations, contribute to desirable flavours in Cheddar cheese (Reddy & Marth, 1993), while excessive lipolysis leads to undesirable or 'rancid' off-flavours.

In this study, the levels of individual FFA from butyric to linoleic acids increased significantly ($P<0{\cdot}05$) throughout the 238 day ripening period. The most notable increases were in palmitic and oleic acids, which in two of the three trials, increased approximately twofold over the ripening period in cheese made with the starter *Lc. lactis* subsp. *cremoris* AM2. A steady increase in the concentrations of individual FFA during Cheddar cheese ripening has been reported previously (Dulley & Grieve, 1974; McNeill & Connolly, 1989; Reddy & Marth, 1993). Marsili (1985) found the best predictors of lipolytic age in Cheddar cheese to be capric, lauric, myristic and palmtic acids; in our study these FFA were also found to increase during ripening. Linolenic acid was not detected in any of the cheeses in the three trials, however this FFA may have been present below the detection level of the analytical method.

At all ripening times, lower levels of individual FFA were found in cheeses from trial 3 than in cheeses from trials 1 or 2. Cheeses of trials 1, 2 and 3 were manufactured in October, November and January, respectively, and it is possible that differences in susceptibility to lipolysis may have occurred as a result of changes in MFGM structure. Indeed previous workers have shown that the pattern of synthesis of fatty acids changes with lactation and that a marked change in MFGM composition occurs during the transition from colostrum to normal milk. (Bauman & Davis, 1974; Eastridge & Palmquist, 1988; Lynch et al. 1992). The influence of seasonality on the FFA composition of cheese was also reported by Macedo & Malcata (1996).

It has long been known that the type of starter strain used for cheese manufacture has a major influence on the development of cheese flavour (Lawrence et al. 1972; Lowrie et al. 1972; Martley & Lawrence, 1972; Visser, 1977). More recently, it has been established that differences in starter cell autolysis influence the level of proteolysis in cheese (Wilkinson et al. 1994*a*, *b*; Crow et al. 1993, 1995; Chapot-Chartier, 1994; O'Donovan et al. 1996). It would appear that starter cell autolysis is a necessary prerequisite to the release of intracellular proteolytic enzymes into cheese curd which subsequently impacts on flavour development and ripening (Crow et al. 1995; Fox et al. 1996*b*; O'Donovan et al. 1996). Lactic acid bacteria (LAB) are generally considered to be weakly lipolytic (Stadhouders & Veringa, 1973; Fox et al. 1993; Chich et al. 1997) and to date, it is not known whether lipolytic activity is influenced by the autolytic properties of the particular strain. In this study, a statistically significant positive relationship was found between FFA levels developed in cheese during ripening and the starter culture used for cheese manufacture. The starter strains used for cheese manufacture were selected on the basis of previously identified differences in their autolytic properties (Wilkinson et al. 1994*b*; 1995). Differences in autolysis of AM2 or HP in cheese as measured by viable cell counts and release of the intracellular marker enzyme LDH agree with previous findings (Martley & Lawrence, 1972; Wilkinson et al. 1994*b*; Chapot-Chartier et al. 1994). Viable cell counts of starter strains are most valid over the first 6–8 weeks of ripening and over this interval the viability of AM2 was much lower than for HP (Crow et al. 1995; O'Donovan et al. 1996). Levels of LDH were also shown to be much higher for AM2 compared with HP confirming the highly autolytic nature of *Lc. lactis* subsp. *cremoris* AM2. FFA results indicated that this highly autolytic strain also developed higher levels of lipolysis. In particular, levels of caprylic ($C_{8:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$) and oleic ($C_{18:0}$) acids were significantly higher ($P<0{\cdot}05$) in cheeses manufactured with *Lc. lactis* subsp. *cremoris* AM2 than in cheeses manufactured using *Lc. lactis* subsp. *cremoris* HP as starter. Assays of lipolytic and esterolytic activities of CFE derived from *Lc. lactis* subsp. *cremoris* AM2 and *Lc. lactis* subsp. *cremoris* HP indicated that differences in lipolysis in cheeses made with these starters were not due to the presence of higher lipolytic and esterolytic activities in strain AM2. Therefore, we suggest that it is likely that these differences in FFA result from a more efficient and extensive release of lipolytic and esterolytic enzymes from the highly autolytic strain *Lc. lactis* subsp. *cremoris* AM2.

Differences in autolysis of AM2 or HP has been attributed to their differing response to the cooking temperatures used in Cheddar cheese manufacture and results in differing viable populations, survival rates and release of intracellular enzymes into cheese (Martley & Lawrence, 1972; Lowrie et al. 1974; Wilkinson et al. 1995). To date, differences in starter autolysis has been shown to result in significant effects on proteolytic events during cheese ripening e.g. reduction in bitterness and increased amino acid production by the highly autolytic starter AM2. Although LAB are weakly lipolytic it appears from the data presented in this study that lipolytic/esterolytic activity may also be influenced by the general mechanism of autolytic release of intracellular enzymes from the starter bacteria in cheese. In an earlier study, which provides some evidence for the effects of autolysis of AM2 on the formation of volatile fat-derived aroma compounds, Walker & Keen (1974) compared the formation of odd numbered ($C:_3$–$C:_{15}$) methyl ketones during the ripening of Cheddar cheese made with strain AM2 or HP. These workers found highest levels of total and individual methyl ketones in cheese made with AM2 compared with HP with differences varying over the 390 d of ripening. Hence, the influence of autolysis of starter bacteria on cheese enzymology and biochemistry may not be confined to proteolysis and differences in the extent of starter autolysis may also affect other enzyme-mediated flavour reactions including the formation of volatile and non-volatile FFA and other important aroma compounds. Future work will therefore concentrate on further elucidation of the nature of the relationship between autolysis and cheese flavour chemistry and the use of fast autolysing starters to accelerate cheese ripening.

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