Lipolysis in Cheddar Cheese Made from Raw, Thermized, and Pasteurized Milks

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ABSTRACT

The evolution of free fatty acids (FFA) was monitored over 168 d of ripening in Cheddar cheeses manufactured from good quality raw milk (RM), thermized milk (TM; 65°C × 15 s), and pasteurized milk (PM; 72°C × 15 s). Heat treatment of the milk reduced the level and diversity of raw milk microflora and extensively or wholly inactivated lipoprotein lipase (LPL) activity. Indigenous milk enzymes or proteases from RM microflora influenced secondary proteolysis in TM and RM cheeses. Differences in FFA in the RM, TM, and PM influenced the levels of FFA in the subsequent cheeses at 1 d, despite significant losses of FFA to the whey during manufacture. Starter esterases appear to be the main contributors of lipolysis in all cheeses, with LPL contributing during production and ripening in RM and, to a lesser extent, in TM cheeses. Indigenous milk microflora and nonstarter lactic acid bacteria appear to have a minor contribution to lipolysis particularly in PM cheeses. Lipolytic activity of starter esterases, LPL, and indigenous raw milk microflora appeared to be limited by substrate accessibility or environmental conditions over ripening.

Key words: Cheddar cheese, lipolysis, heat treatment

INTRODUCTION

It is generally accepted that Cheddar cheeses made from pasteurized milk tend to develop a less intense flavor and ripen more slowly than cheeses made from raw milk (McSweeney et al., 1993; Shakeel-Ur-Rehman et al., 2000a,b). This outcome is thought to result from inactivation of indigenous milk enzymes and destruction of the thermolabile microflora by pasteurization.

The agents responsible for lipolysis in raw milk Cheddar cheese are indigenous milk lipoprotein lipase (LPL; EC 3.1.1.3.4), raw milk microflora, starter lactic acid bacteria, and nonstarter lactic acid bacteria (NSLAB;

Collins et al., 2003; Beuvier and Buchin, 2004). In the northeastern United States and Canada, most Cheddar cheese is manufactured from high quality Grade A milk that has been thermized (Lau et al., 1991; Roy et al., 1997). Thermization is a generic description of a range of subpasteurization heat treatments (57 to 68°C × 10 to 20 s) that markedly reduce the number of spoilage bacteria in milk with minimal heat damage (Stepaniak and Rukke, 2003).

Studies comparing Cheddar cheeses made from raw, pasteurized, and microfiltered milk (McSweeney et al., 1993), blends of raw and pasteurized milks (Shakeel-Ur-Rehman et al., 2000a), and raw and pasteurized cheese ripened at different temperatures (Shakeel-Ur-Rehman et al., 2000b) indicate that higher levels of lipolysis in raw milk Cheddar cheeses may be due to differing growth rates of NSLAB. In cheeses made from raw milk, NSLAB populations increase and generally reach a plateau more rapidly compared with pasteurized milk cheeses. The NSLAB species in the resulting cheeses also appear to differ with severity of heat treatment of the milk (McSweeney et al., 1993; Roy et al., 1997; Beuvier and Buchin, 2004). The microflora of Cheddar cheese has been shown to comprise species other than NSLAB, including enterococci and psychrotrophic bacteria, which may also contribute to lipolysis in Cheddar cheese (Dovat et al., 1970; Ouattara et al., 2004).

Despite the numerous studies comparing lipolysis in raw and pasteurized Cheddar cheese, most studies report the extent of total lipolysis without supplying quantitative data on the profiles and levels of individual FFA generated during ripening. Moreover, there are no reports in the literature on the effect of thermization on lipolysis in Cheddar cheese. This study was therefore undertaken to provide quantitative data on lipolysis during ripening in Cheddar cheeses made from raw, thermized, and pasteurized milks.

MATERIALS AND METHODS

Cheese Manufacture

Lactococcus lactis ssp. *lactis* 303 obtained from Chr. Hansen Ireland Ltd. (Little Island, Co. Cork, Ireland)

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was grown overnight at 23°C in heat-treated (95°C for 30 min) 10% (wt/vol) reconstituted skim milk powder (Golden Vale Food Products Ltd., Co. Cork, Ireland). This strain was used as starter culture at a level of 1.5% (wt/wt).

Cheddar cheeses were manufactured in 500-L vats on 3 separate occasions using milk obtained from the Friesian herd at the Dairy Production Centre, Moorepark, Ireland. For each cheese trial, approximately 1,500 L of milk was collected from the farm the day before manufacturing and held overnight at 4°C. On the day of cheese manufacture the milk was heated to ~30°C, approximately 500 L was then passed through the pasteurizer and heated to 72°C × 15 s, and then pumped into the first vat for pasteurized milk (PM) cheeses. For thermized milk (TM) cheeses, ~500 L of the raw milk was passed through the pasteurizer, heated to 65°C × 15 s, and pumped into the second vat. For the raw milk (RM) cheeses, the milk was pumped through the pasteurizer at ambient temperature and into the third vat. Before cheese manufacture, milk from the herd was analyzed to ensure the absence of *Escherichia coli*, fecal streptococci, *Salmonella* spp. and *Listeria monocytogenes*. Milks were not standardized for cheese making. Cheeses were manufactured using conventional cheese-making methods; curd was cooked to 38.5°C, pitched at pH 6.15, milled at pH 5.35, and salted at 2.7% (wt/wt). All practical precautions were undertaken during manufacturing to prevent cross-contamination between vats. Cheeses were ripened at 8°C for 168 d and sampled at 14 d of ripening for compositional analysis; all other analyses were carried out at d 1, 14, 28, 56, 112, and 168.

Chemical Composition of Milk

Milks of all types were analyzed for fat (IDF, 1996), protein (IDF, 2001), and casein (IDF, 2004). Lactose was measured on a Milkoscan 605 (Foss Electric, Hillerød, Denmark). All analyses were conducted in duplicate.

Microbiological Quality of Milk

Coliforms in RM, TM, and PM were enumerated on violet red bile agar (Oxoid, Basingstoke, UK), incubated at 30°C for 24 h; total bacterial count (TBC) was evaluated on milk plate count agar (Oxoid) incubated at 30°C for 72 h. Psychrotrophic bacterial count (PBC) was enumerated on milk plate count agar (Oxoid) incubated at 8°C for 10 d, enterococci were enumerated on kanamycin esculin azide agar (Merck, Darmstadt, Germany) incubated at 37°C for 24 h, and NSLAB were incubated anaerobically on LBS agar (Rogosa et al., 1951) at 30°C

for 5 d. Staphylococci were enumerated on Baird Parker agar (Merck) with egg yolk tellurite supplement (Merck) and incubated at 37°C for 48 h. *Staphylococcus aureus* were identified on Baird Parker agar as black colonies surrounded by a clear zone and that tested positive for coagulase using the staphylase test (Oxoid). Lipolytic bacteria were estimated on tributyrin, triolein, and butterfat agars (Merck) incubated at 8 and 30°C for 10 and 3 d, respectively. Lipolytic colonies were identified as colonies surrounded by clear zones in an otherwise turbid culture medium. Somatic cell count of RM was measured on a Bentley Somacount 300 (Bentley Instrument Inc., Chaska, MN). All analyses were carried out in duplicate.

Enzyme Activity of Milks

Commercially available API-ZYM kits (BioMérieux, Marcy-L'Etoile, France) for semiquantitative assay of 19 enzyme activities were used to monitor the effect of the milk heat treatments on a range of enzyme activities. Raw milk, TM, and PM (65 µL) were inoculated into API-ZYM strips according to the manufacturer's instructions and incubated at 37°C for 5 h before color was developed using ZYM A and ZYM B reagents. The intensity of color developed within 5 min was compared with the API-ZYM color reaction chart and the enzyme activity was graded from 0 to 5. A value of 0 corresponded to negative reaction and 5 to a reaction of maximum intensity. All analysis was conducted in duplicate.

Lipolytic Activity of Cheese Milks

Aseptically drawn RM, TM, and PM samples with 0.05% added sodium azide, were incubated at 37°C for 15 h with agitation (100 rpm). Individual FFA (C_{4:0} to C_{18:1}) in milk samples were quantified before and after incubation by gas chromatograph flame-ionized detection according to the method described by Hickey et al. (2006). All analyses were conducted in duplicate.

Cheese Composition

Grated cheese samples were analyzed in duplicate for pH, moisture, fat, salt, and total nitrogen as described by Hickey et al. (2006).

Microbiological Analysis of Cheese

Microbiological analysis of the cheese was carried out in duplicate at each sampling point. Starter lactic acid bacteria were enumerated on LM17 agar after 3 d at 30°C over the first 56 d of ripening (Terzaghi and Sandine, 1975). Nonstarter lactic acid bacteria, coliforms,

enterococci, staphylococci, TBC, psychrotrophic bacteria, and lipolytic bacteria were enumerated periodically over the 168-d ripening period.

Starter Autolysis in Cheese

Autolysis of starter culture in cheese during ripening was monitored periodically over the first 56 d by assaying the juice for the activity of the intracellular enzyme lactate dehydrogenase (**LDH**, EC 1.1.1.27). Cheese juice was extracted from cheeses as described by Wilkinson et al. (1994) and assayed immediately for LDH activity by a modification of the method of Wittenberger and Angelo (1970) by measuring the decrease in absorbance at 340 nm (Spectronic Genesys 5 spectrophotometer, Milton Roy Company, Rochester, NY) resulting from the pyruvate-dependent oxidation of NADH in the presence of fructose-1,6 bis-phosphate. Results were expressed in LDH units, where one unit was defined as the amount of enzyme that catalyzes the oxidation of one micromole of NADH per minute per milliliter of cheese juice.

Assessment of Proteolysis in Cheese During Ripening

Proteolysis in cheeses was monitored by measuring the percentage of total N soluble at pH 4.6 (**pH 4.6-SN**) and in 5% phosphotungstic acid (**PTA-SN**) and individual free amino acids (**FAA**) as described by Hickey et al. (2006). All analyses were conducted in triplicate.

Assessment of Lipolysis in Cheese During Ripening

Individual FFA ($C_{4:0}$ to $C_{18:1}$) in cheese samples were quantified by gas chromatograph flame-ionized detection according to Hickey et al. (2006). All analysis was conducted in triplicate.

Statistical Analysis

A randomized complete block design, which incorporated the 3 treatments (heat treatments) and 3 blocks (replicate trials), was used for analysis of the response variables relating to milk (chemical and FFA) and cheese composition. Analysis of variance was carried out using the GLM procedure of SAS (version 9.1.3, SAS Institute Inc., 2003) in which the effect of treatment and replicates were estimated for all response variables. Duncan's multiple-comparison test was used as a guide for pair comparison of the treatment means. The level of significance was determined at $P < 0.05$.

A split-plot design was used to monitor the effect of treatment, ripening time, and their interaction on the

response variables measured at regular intervals during ripening; that is, LDH activity, pH4.6-SN, 5% PTA-SN, FAA, and FFA. The ANOVA for the split plot was carried out using the GLM procedure of SAS (SAS Institute, 2003). Statistically significant differences ($P < 0.05$) between the different treatments were determined by Fisher's least significant difference test.

Data relating to enzyme activities monitored by API-ZYM assay were not statistically analyzed but are included as observations.

RESULTS AND DISCUSSION

Composition of Milks

Heat treatment had no significant effect on the composition of RM, TM, and PM. The mean protein, fat, lactose concentrations, and casein number (casein N $\times$ 100/total N) were 3.6, 3.9, 4.5, and 79%, respectively (data not shown).

Bacteriological Quality of Cheese Milks

Raw milk was of good microbial quality: TBC and SCC were within normal ranges for Cheddar cheese manufacture (European Union, 1992; Table 1). In agreement with Gilmour et al. (1981), thermization reduced TBC, PBC, coliforms, enterococci, NSLAB, and staphylococci and eliminated coagulase-positive staphylococci. Similar to the findings of Driessen and Stadhouders (1975), lipolytic bacteria were not detected in TM (Table 1). In agreement with Beuvier et al. (1997) and Shakeel-Ur-Rehman et al. (2000a,b), pasteurization further reduced TBC, PBC, and NSLAB and eliminated coliforms, enterococci, and staphylococci. Very low numbers (<1 log cfu/mL) of lipolytic bacteria were detected in PM, which was probably due to postpasteurization contamination.

Enzymatic Activity of Milk

Enzymatic activities of RM, TM, and PM are shown in Table 2. Similar to the results of Griffiths (1986), leucine arylamidase was completely inactivated by thermization, which also inactivated esterase activity. As expected, pasteurization completely inactivated alkaline phosphatase, an indicator of pasteurization (Stepaniak and Rukke, 2003), and also inactivated most enzyme activities that could be detected using the API-ZYM kit.

Cheese Composition

Heat treatment of the milks had no significant effect on gross cheese composition, which was within the

Table 1. Bacterial count (log cfu/mL) and SCC of raw (RM), thermized (TM), and pasteurized (PM) milks^{1,2}

Bacteria enumerated	RM	TM	PM
Total bacterial count	4.34 (0.50)	3.70 (0.92)	2.82 (0.72)
Coliforms	1.96 (0.52)	0.20 (0.35)	ND
Enterococci	3.11 (1.26)	1.15 (0.16)	ND
Psychrotrophic count	4.21 (0.75)	2.88 (1.81)	1.49 (0.66)
Nonstarter lactic acid bacteria	2.32 (0.52)	0.67 (0.50)	0.08 (0.14)
Total staphylococci	2.34 (1.06)	0.17 (0.29)	ND
Coagulase-positive staphylococci	1.73 (1.52)	ND	ND
Lipolytic count at 30°C			
Triolein-hydrolyzing	ND	ND	0.83 (1.43)
Tributyrin-hydrolyzing	1.96 (2.01)	ND	0.92 (1.30)
Butterfat-hydrolyzing	ND	ND	ND
Lipolytic count at 8°C			
Triolein-hydrolyzing	ND	ND	ND
Tributyrin-hydrolyzing	ND	ND	ND
Butterfat-hydrolyzing	ND	ND	ND
SCC, cells/mL	210 × 10 ³		

¹Numbers represent means (SD; n = 3); ND = Not detected.

²Higher SD than the means occurred when the microbial group was absent from 1 or 2 of the triplicate trials.

ranges recommended by Gilles and Lawrence (1973) for good quality Cheddar (Table 3).

Bacteriological Quality of Cheeses

Microbial counts in RM, TM, and PM cheeses during ripening are shown in Table 4. In agreement with previous studies (O'Donovan et al., 1996; Hickey et al., 2006; Sheehan et al., 2006), the viability of starter *L. lactis*

303 remained at ~9 log cfu/g during the early stages of ripening and was similar in all cheeses.

At 1 d of ripening, NSLAB numbers were 1.1, 1.9, and 4.2 log cfu/g and increased rapidly over the first 56 d reaching 5.7, 6.0, and 7.6 log cfu/g in PM, TM, and RM cheeses, respectively. After 56 d, NSLAB reached a plateau in RM cheeses, whereas NSLAB continued to increase (albeit at a slower rate) in TM and PM cheeses. At 168 d of ripening, NSLAB populations had reached 7.2, 7.6, and 7.6 log cfu/g in PM, TM, and RM cheeses, respectively.

Low numbers of coliforms were present in all cheeses at d 1 and declined during ripening. Enterococcal populations remained relatively stable during ripening in all cheeses and at 168 d were present at 5.5, 4.0, and 1.0 log cfu/g in RM, TM, and PM cheeses, respectively.

Table 2. Enzyme activity after heat treatment of raw (RM), thermized (TM), and pasteurized (PM) milks detected using the API-ZYM system¹

Enzyme activity	Observed color intensity ²		
	RM	TM	PM
Alkaline phosphatase	5	5	0
Esterase (C _{4:0})	2	0	0
Esterase/lipase (C _{8:0})	2	0	0
Lipase (C _{14:0})	0	0	0
Leucine arylamidase	2	0	0
Valine arylamidase	0	0	0
Cystine arylamidase	0	0	0
Trypsin	0	0	0
β-Chymotrypsin	0	0	0
Acid phosphatase	4	4	1
Naphthol-AS-bi-phosphohydrolase	4	3	1
α-Galactosidase	0	0	0
β-Galactosidase	0	0	0
β-Glucuronidase	0	0	0
α-Glucosidase	0	0	0
β-Glucosidase	0	0	0
N-Acetyl-β-glucosaminidase	0	0	0
α-Mannosidase	1	0	0
α-Fucosidase	0	0	0

¹Data presented are the means of triplicate trials.

²Color intensity: 0 = negative reaction; 5 = reaction of maximum intensity.

Table 3. Composition of Cheddar cheeses (% wt/wt) made from raw (RM), thermized (TM), and pasteurized (PM) milks¹

Composition ²	Cheese			SED
	RM	TM	PM	
Moisture	36.5 ^a	36.5 ^a	37.0 ^a	0.35
Fat	32.9 ^a	32.7 ^a	32.4 ^a	0.16
Protein	25.7 ^a	25.2 ^a	25.3 ^a	0.30
Salt	1.6 ^a	1.6 ^a	1.7 ^a	0.34
S/M	4.5 ^a	4.4 ^a	4.5 ^a	0.37
FDM	51.7 ^a	51.5 ^a	51.4 ^a	0.37
MNFS	54.4 ^a	54.2 ^a	54.7	0.52
pH	5.1 ^a	5.1 ^a	5.1 ^a	0.06

^aValues within rows not sharing a common superscript differ significantly, *P* < 0.05.

¹Data presented are the means of triplicate trials; SED = standard error of difference across the treatment means.

²S/M = Salt in moisture; FDM = fat in DM; MNFS = moisture in nonfat substrates.

Table 4. Starter, adventitious, and lipolytic bacterial counts (log cfu/g) present in Cheddar cheeses made from raw (RM), thermized (TM), and pasteurized (PM) milk during ripening^{1,2}

Cheese	Ripening time, d	Starter	Adventitious bacteria					Lipolytic bacteria ³		
			NSLAB ⁴	Coliforms	Enterococci	Total staphylococci	Coagulase-positive staphylococci	Tributylin, 30°C	Triolein, 30°C	Butterfat, 30°C
RM	1	9.26 (0.58)	4.21 (0.59)	2.98 (1.45)	4.45 (0.18)	5.01 (0.07)	4.34 (0.11)	4.59 (3.32)	ND ⁵	ND
	14	9.20 (0.28)	5.66 (0.63)	3.19 (0.10)	5.31 (1.38)	5.15 (0.57)	4.48 (0.03)	3.31 (1.02)	ND	ND
	28	9.17 (0.18)	6.49 (0.45)	2.90 (0.21)	5.53 (0.91)	4.90 (0.04)	4.56 (0.13)	3.46 (0.65)	ND	ND
	56	9.08 (0.28)	7.58 (0.73)	2.40 (0.10)	5.42 (1.32)	4.95 (0.06)	3.09 (2.68)	4.12 (0.31)	ND	ND
	112	—	7.54 (0.13)	0.90 (0.78)	5.49 (1.31)	4.33 (0.46)	3.85 (0.40)	4.60 (2.05)	ND	ND
	168	—	7.63 (0.40)	ND	5.49 (1.33)	4.22 (0.77)	4.02 (0.84)	2.95 (3.43)	ND	ND
TM	1	9.44 (0.55)	1.94 (1.06)	1.43 (1.49)	2.77 (0.20)	2.87 (1.01)	ND	1.46 (1.36)	ND	ND
	14	9.09 (0.41)	3.70 (0.43)	1.20 (1.22)	3.86 (1.64)	3.66 (1.07)	ND	1.37 (1.42)	ND	ND
	28	9.03 (0.33)	4.82 (1.11)	1.31 (1.26)	4.03 (1.39)	3.51 (1.50)	ND	1.81 (1.57)	ND	ND
	56	9.02 (0.48)	6.00 (0.88)	1.54 (1.04)	4.01 (1.76)	2.62 (0.76)	ND	1.74 (1.54)	ND	ND
	112	—	7.41 (0.36)	ND	3.85 (1.78)	3.16 (0.42)	ND	1.77 (1.54)	ND	ND
	168	—	7.60 (0.16)	ND	3.96 (1.67)	1.47 (1.69)	ND	2.67 (2.68)	ND	ND
PM	1	9.50 (0.19)	1.09 (1.14)	1.81 (0.65)	0.82 (0.91)	ND	ND	0.22 (0.38)	ND	ND
	14	9.38 (0.09)	2.77 (1.30)	0.51 (0.88)	1.34 (0.71)	ND	ND	ND	ND	ND
	28	9.39 (0.29)	4.43 (2.21)	0.60 (1.04)	0.70 (0.81)	ND	ND	0.60 (1.04)	ND	ND
	56	9.43 (0.36)	5.69 (0.58)	0.95 (1.35)	1.19 (1.03)	ND	ND	1.17 (1.03)	ND	ND
	112	—	5.71 (1.21)	ND	1.04 (0.92)	ND	ND	0.33 (0.56)	ND	ND
	168	—	7.15 (0.68)	ND	1.03 (0.98)	ND	ND	0.22 (0.38)	ND	ND

¹Numbers represent means (SD; n = 3).²Higher SD than the means occurred when the microbial group was absent from 1 or 2 of the triplicate cheese trials.³No lipolytic bacteria were detected on tributyrin, triolein, and butterfat agar incubated at 8°C.⁴NSLAB = Nonstarter lactic acid bacteria.⁵ND = Not detected.

Staphylococci were absent from PM cheeses but were present at 5.0 and 2.8 log cfu/g at 1 d, and at 4.2 and 1.5 log cfu/g at 168 d in RM and TM cheeses, respectively. Throughout ripening coagulase-positive staphylococci accounted for between 60 and 95% of the staphylococci detected in RM cheeses, indicative of the presence of *Staphylococcus aureus*.

Tributylin-hydrolyzing bacteria capable of growth at 30°C were detected at 4.6, 1.5, and 0.2 log cfu/g at 1 d and at 3.0, 2.7, and 0.2 log cfu/g at 168 d in RM, TM, and PM cheeses, respectively. However, when plates were incubated at 8°C, tributyrin-hydrolyzing bacteria were not detected in any of the cheeses. Triolein- or butterfat-hydrolyzing bacteria were not detected in any of the cheeses during ripening when agar plates were incubated at 8 or 30°C. The majority of lipolytic colonies randomly isolated from RM, TM, and PM tributyrin agar plates at 28 d were identified as gram-positive, catalase-positive cocci in RM and TM cheeses, and gram-positive rods in PM cheeses. Lipolytic bacteria previously isolated from Cheddar cheese belong predominantly to the genus *Staphylococcus* (Franklin and Sharpe, 1963; Gillies, 1971). In this study no correlation was observed between numbers of tributyrin-hydrolyzing bacteria detected and staphylococcal populations.

In agreement with Martley and Crow (1993), it is likely that NSLAB and other adventitious microflora in

PM cheeses entered the cheese from the environment, cheese-making equipment, or personnel during manufacture, whereas the more diverse adventitious bacteria in RM and TM cheeses would likely have originated from the milk (McSweeney et al., 1993).

Starter Autolysis

Increases in autolysis as detected by released LDH activity in cheese juice were monitored during the first 56 d of ripening (data not shown) when the starter lactic acid bacteria were the predominant population. No significant differences were observed in the LDH activity between the cheeses, but in agreement with O'Donovan et al. (1996), Hickey et al. (2006), and Sheehan et al. (2006), LDH activity increased significantly ($P < 0.001$) over ripening.

Proteolysis

In all cheeses, pH 4.6-SN, 5% PTA-SN, and FAA increased significantly ($P < 0.001$) during ripening. Similar to results of previous studies (McSweeney et al., 1993; Shakeel-Ur-Rehman et al., 1999, 2000a,b), pasteurization of milk did not significantly affect the levels of primary proteolysis (pH 4.6-SN) during ripening (data not shown). Significant differences were not ob-

Figure 1. Proteolytic indices: A) 5% phosphotungstic acid-soluble N (PTA-SN) as a percentage of total N (TN), and B) total FAA in Cheddar cheeses made from raw (■), thermized (●), and pasteurized (▲) milks. Error bars indicate ± 1 SD ($n = 3$).

served between TM and RM cheeses for primary proteolysis as indicated by pH 4.6-SN. Similar to the results of Roy et al. (1997), levels of primary proteolysis were similar in TM and PM cheeses. In agreement with O'Keeffe et al. (1978), rennet appeared to be the principal agent responsible for primary proteolysis in all cheeses. Similar to trends noted for pH 4.6-SN, levels of 5% PTA-SN were not significantly affected by severity of milk heat treatment (Figure 1). Therefore, it appears that in this study starter peptidases were the main contributors to formation of 5% PTA-SN; however, at the latter stages of ripening, levels of 5% PTA-SN were numerically higher in TM and RM cheeses, which may be due to the contribution of peptidases originating from the indigenous milk microflora or residual activity of indigenous milk proteinases.

Mean levels of FAA increased significantly ($P < 0.001$) in the order PM < TM < RM (Figure 1) and are in agreement with trends noted by Shakeel-Ur-Rehman et al. (1999, 2000a,b), who reported higher levels of FAA in RM compared with PM cheeses. Levels of FAA were similar in all cheeses up to 28 d; thereafter, levels

of FAA were significantly higher ($P < 0.05$) in RM cheeses compared with PM and TM cheeses. Thermized milk and PM cheeses had similar levels of FAA up to 56 d; thereafter, levels were significantly higher ($P < 0.05$) in TM cheeses. The greater accumulation of FAA in TM and RM cheeses (TM < RM) may reflect the contribution of peptidases from indigenous microflora (Jensen et al., 1975; McSweeney et al., 1993). McSweeney et al. (1993) and Shakeel-Ur-Rehman et al. (1999, 2000a,b) suggested that peptidases from NSLAB might be responsible for higher levels of FAA in RM cheeses. Shakeel-Ur-Rehman et al. (2000a) suggested that, in addition to the numbers of NSLAB present in RM cheeses, the particular NSLAB strains might also influence FAA formation. The higher level of FAA in TM and RM cheeses might also be due to numerically higher levels of 5% PTA-SN, which may reflect slight differences in levels of substrate for starter peptidases.

During ripening, mean levels of most individual FAA were significantly influenced by heat treatment except for Arg, Cys, Pro, and Ser (data not shown). Mean levels of Ala, Asp, Leu, Phe, and Thr were similar but significantly ($P < 0.05$) lower in TM and PM cheeses compared with RM cheeses. Mean levels of Glu, Gly, His, Ile, Lys, Met, and Val were significantly ($P < 0.05$) higher in RM compared with TM cheeses, which in turn were significantly higher than those in PM cheeses.

FFA Profile and Lipolytic Activity in Cheese Milks

The FFA profiles of the milks before and after incubation are shown in Table 5. Thermized milk and PM had similar and significantly lower ($P < 0.05$) levels of short-chain FFA (SCFFA, $C_{4:0}$ to $C_{8:0}$) and medium-chain FFA (MCFFA, $C_{10:0}$ to $C_{14:0}$) compared with RM. Significant differences were not observed between milks for long-chain FFA (LCFFA, $C_{16:0}$ to $C_{18:1}$). Overall, FFA were 43 and 50% higher in RM than in TM and PM, respectively. The higher levels FFA in RM were most likely due to lipolysis by LPL during the pumping of the RM through the pasteurizer and into the vat because such conditions are known to enhance the action of LPL (Downey, 1980) by causing damage to the milk fat globule membrane and exposing the fat substrate.

Incubation of RM, TM, and PM to ascertain indigenous lipolytic activity resulted in a 6-, 5-, and 1.5-fold increase in the levels of total FFA (TFFA; Table 5). Increases in the levels of FFA in RM and TM during incubation was most likely due to LPL activity, with lower levels observed in TM as a result of partial inactivation of LPL. Although a significant increase in the levels of FFA was observed for PM after incubation, it was much lower than in TM and RM and may have

Table 5. Concentrations of FFA (mg/kg of milk) in raw (RM), thermized (TM), and pasteurized (PM) milks before and after 15 h of incubation¹

FFA ²	RM		TM		PM	
	0 h	15 h	0 h	15 h	0 h	15 h
C _{4:0}	15 (7) ^b	138 (32) ^a	5 (12) ^a	30 (22) ^a	4 (1) ^a	4 (1) ^a
C _{6:0}	10 (5) ^b	95 (24) ^a	4 (1) ^a	23 (19) ^a	2 (2) ^a	3 (0) ^a
C _{8:0}	10 (4) ^b	60 (15) ^a	4 (0) ^a	20 (14) ^a	3 (0) ^a	3 (0) ^a
C _{10:0}	14 (6) ^b	90 (30) ^a	6 (1) ^a	29 (22) ^a	5 (1) ^a	5 (1) ^a
C _{12:0}	14 (5) ^b	77 (22) ^a	7 (1) ^a	26 (14) ^a	6 (1) ^a	6 (0) ^a
C _{14:0}	28 (10) ^b	158 (19) ^a	14 (4) ^b	54 (20) ^a	12 (1) ^a	14 (1) ^a
C _{16:0}	62 (13) ^b	360 (79) ^a	45 (6) ^b	226 (99) ^a	41 (15) ^b	67 (2) ^a
C _{18:0}	13 (10) ^a	104 (62) ^a	13 (3) ^a	51 (9) ^a	14 (2) ^a	22 (1) ^a
C _{18:1}	57 (30) ^a	250 (126) ^a	32 (24) ^a	129 (81) ^a	26 (7) ^a	52 (3) ^a
SCFFA (sum of C _{4:0} to C _{8:0})	35 (16) ^b	293 (70) ^a	12 (2) ^a	73 (74) ^a	9 (1) ^a	10 (1) ^a
MCFFA (sum of C _{10:0} to C _{14:0})	56 (21) ^b	325 (71) ^a	26 (6) ^a	109 (55) ^a	21 (1) ^a	24 (1) ^a
LCFFA (sum of C _{16:0} to C _{18:1})	133 (52) ^b	714 (262) ^a	90 (33) ^a	406 (186) ^a	81 (19) ^a	137 (7) ^b
Total (sum of C _{4:0} to C _{18:1})	224 (88) ^b	1331 (395) ^a	128 (39) ^a	588 (294) ^a	111 (24) ^b	173 (6) ^a

^{a,b}Values within rows not sharing a common superscript differ significantly, $P < 0.05$.

¹Numbers represent means (SD; $n = 3$).

²SCFFA = Short-chain FFA; MCFFA = medium-chain FFA; LCFFA = long-chain FFA.

arisen from physical agitation of the milks during incubation (Hadland and Hoye, 1974). Indeed it is well recognized that pasteurization extensively inactivates LPL: Andrews et al. (1987) found that heating milk for 15 s at 70 and 75°C resulted in residual LPL activities of 2 and 0%, respectively. It is unlikely that heat-resistant bacterial lipases contributed to the production of FFA in RM, TM, or PM because psychrotrophic bacteria need to reach populations of $\sim 10^6$ to 10^7 cfu/mL before significant lipolytic activity occurs (Ouattara et al., 2004). Psychrotrophic bacteria were at 10^4 cfu/mL in RM before incubation and sodium azide was included to inhibit bacterial growth (Table 1).

Lipolysis

Comparison of samples of RM, TM, and PM with their equivalent cheeses at d 1 indicated that approximately 50, 26, and 22% of TFFA present in the RM, TM, and PM, respectively, were lost during the Cheddar cheese manufacturing process (data not shown). During manufacture of RM, TM, and PM cheeses, approximately 91, 78, and 77% of the SCFFA, 47, 7, and 3% of the MCFFA, and 47, 24, and 27% of the LCFFA were lost to the whey, respectively. Loss of SCFFA and MCFFA to the whey was probably related to their water solubility. Higher losses of SCFFA, MCFFA, and LCFFA in the RM cheeses may be due to the higher level of these FFA in RM.

At d 1, levels of FFA in PM and TM cheeses were similar and significantly lower ($P < 0.05$) than in RM cheeses (Table 6); no significant differences were observed between TM and PM cheeses. The higher levels

of FFA in RM cheeses at d 1 probably originated from higher levels of FFA in the milk and possibly LPL activity in the vat. It is unlikely that RM microflora contributed to lipolysis during cheese manufacture in the vat because their numbers were thought to be low. In addition, esterases from psychrotrophic bacteria were not detected on tributyrin, triolein, or butterfat agar incubated at 8°C.

Mean levels of FFA increased significantly over ripening in all cheeses (Table 6). Mean levels of TFFA were significantly higher in RM cheeses compared with PM cheeses; however, no significant differences in TFFA were observed between TM and RM cheeses or between TM and PM cheeses. The higher levels of TFFA in RM Cheddar cheeses are in agreement with trends noted in previous studies (McSweeney et al., 1993; Shakeel-Ur-Rehman et al., 2000a,b). The rate of increase of SCFFA, MCFFA, and LCFFA were compared during ripening and no significant differences were observed for the rate of increase of MCFFA or LCFFA; however, the mean rate of increase of SCFFA increased significantly ($P < 0.05$) in the order RM < TM < PM.

Some interesting differences were noted between SCFFA, MCFFA, and LCFFA in PM, TM, and RM cheeses over ripening. The percentage increase of SCFFA and MCFFA was in the order of PM < TM < RM, with the LCFFA in the order of RM < TM < PM. However, SCFFA, MCFFA, and LCFFA numerically increased in the order of RM < TM < PM. This suggests that esterase activity predominated in PM cheeses and was most likely due to the starter bacteria (*L. lactis*, 303) because levels of NSLAB and other indigenous microflora did not reach sufficient numbers at all or

Table 6. Free fatty acid (mg/kg of cheese) composition (C_{4:0} to C_{18:1}) and ANOVA of raw (RM), thermized (TM), and pasteurized (PM) milk Cheddar cheeses during ripening¹

Cheese	Ripening time, d	FFA									Total
		C _{4:0}	C _{6:0}	C _{8:0}	C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}	C _{18:0}	C _{18:1}	
RM	1	4 (1)	7 (2)	14 (4)	36 (4)	52 (6)	150 (26)	306 (32)	98 (11)	211 (8)	878 (72)
	14	7 (2)	8 (1)	16 (1)	34 (11)	53 (11)	164 (22)	311 (43)	100 (7)	206 (4)	898 (95)
	28	11 (3)	7 (1)	11 (1)	48 (6)	74 (7)	132 (11)	379 (35)	88 (3)	202 (11)	953 (54)
	56	16 (3)	7 (1)	12 (0)	50 (4)	78 (1)	167 (15)	360 (46)	92 (6)	222 (6)	1005 (58)
	112	27 (5)	10 (2)	14 (2)	50 (8)	78 (2)	178 (14)	360 (44)	90 (10)	253 (22)	1058 (79)
	168	39 (1)	14 (1)	15 (10)	57 (4)	73 (15)	211 (16)	420 (60)	110 (5)	283 (31)	1223 (108)
TM	1	3 (2)	6 (0)	12 (2)	28 (9)	40 (8)	127 (16)	262 (23)	90 (9)	194 (17)	762 (62)
	14	6 (0)	7 (1)	13 (3)	29 (8)	42 (7)	136 (10)	277 (21)	91 (8)	200 (29)	801 (57)
	28	9 (0)	6 (1)	9 (0)	41 (2)	62 (2)	109 (5)	322 (18)	70 (6)	178 (20)	805 (35)
	56	13 (2)	6 (1)	10 (1)	42 (1)	66 (2)	137 (23)	303 (13)	77 (7)	199 (32)	854 (55)
	112	23 (2)	8 (1)	11 (1)	43 (4)	68 (6)	155 (9)	318 (27)	74 (16)	221 (9)	920 (37)
	168	34 (4)	10 (1)	12 (1)	46 (0)	65 (2)	171 (9)	341 (17)	89 (11)	215 (27)	984 (57)
PM	1	1 (0)	4 (0)	11 (1)	18 (4)	30 (3)	114 (8)	224 (14)	87 (6)	183 (22)	673 (43)
	14	4 (1)	5 (0)	12 (1)	18 (5)	33 (6)	119 (4)	229 (10)	87 (6)	173 (13)	681 (20)
	28	8 (0)	4 (1)	8 (0)	36 (3)	58 (3)	93 (2)	269 (28)	71 (6)	158 (20)	705 (34)
	56	12 (1)	4 (1)	9 (1)	38 (3)	61 (6)	123 (22)	259 (11)	71 (11)	178 (26)	755 (54)
	112	20 (1)	6 (0)	9 (1)	36 (3)	58 (4)	131 (3)	266 (14)	63 (20)	180 (21)	770 (35)
	168	30 (3)	8 (1)	10 (1)	40 (3)	57 (1)	146 (9)	291 (18)	78 (10)	196 (29)	856 (51)
ANOVA											
Treatment ²		NS	*	*	*	*	*	*	*	*	*
Ripening time		***	***	***	***	***	***	***	***	***	***
Treatment × ripening time		NS	NS	NS	NS	NS	NS	***	NS	***	***

¹Numbers represent means (SD; n = 3).²Treatment = Raw, thermized, or pasteurized milk.**P* < 0.05; ****P* < 0.001.

until late in ripening, and LPL activity appeared to be essentially inactivated by pasteurization at 72°C × 15 s. However, the influence of other sources of lipolytic activity appears to increase in TM and in particular RM cheeses, due to increases in FFA content and to differences in the ratio of SCFFA, MCFFA, and LCFFA. The increase in MCFFA and LCFFA in TM and RM cheeses is most likely due to LPL activity because LPL is active toward tri- and diacylglycerols and has *sn*-1 and *sn*-3 specificity and will therefore cleave longer chain FFA (Reiter et al., 1969; Olivecrona and Bengtsson-Olivecrona, 1991). However, NSLAB (McSweeney et al., 1993; Shakeel-Ur-Rehman et al., 1999, 2000a,b) and indigenous microflora (Dovat et al., 1970; Gillies, 1971) might also contribute to lipolysis due to their high numbers, but because they have only esterase activity, they are unlikely to be responsible for the increase in MCFFA and LCFFA over ripening. Changes to the milk fat substrate induced by heat treatment (Dalglish and Banks, 1991; Lopez et al., accepted) might also have influenced the accessibility of fat substrate to lipases and esterases during ripening of PM and TM cheeses.

In relation to individual FFA, mean levels of C_{4:0} were similar in all cheeses during ripening indicating that the starter (*L. lactis* 303) was the most likely source for its production because it was the only lipolytic source

that was similar in all cheeses. Mean levels of C_{8:0}, C_{10:0}, C_{14:0}, C_{18:0}, and C_{18:1} were similar in TM and PM cheeses but significantly lower compared with RM cheeses. Raw milk cheeses had significantly higher mean levels of C_{6:0}, C_{12:0}, and C_{16:0} compared with PM cheeses but no significant differences were observed between RM and TM cheeses for these FFA (Table 6). It is interesting to note that significant decreases (*P* < 0.001) in mean levels of C_{6:0}, C_{8:0}, C_{14:0}, and C_{18:0} were observed in all cheeses during the first 28 d of ripening. This may have been due to catabolism of FFA (Collins et al., 2003).

The overall increase in FFA during ripening was modest in all cheeses compared with the increase in FFA, despite the potential lipolytic sources. The modest extent of lipolysis may be due to 1) immobilization of LPL in the casein network during cheese manufacture (Fox and Stepaniak, 1993); 2) a reduction in LPL activity during cheese manufacturing as a result of pH decrease or removal of LPL or LPL activators in the whey (Geurts et al., 2003; Choisy et al., 2000); 3) a lack of suitable mono- and diacylglycerols substrates for starter and NSLAB esterases (Holland et al., 2005); 4) the physical state of the milkfat substrate during ripening (Carunchia Whetstine et al., 2006); or 5) absorption of caseins and whey proteins onto the surface of fat globules during cheese production (Michalski et

al., 2001; Lopez et al., accepted) thereby limiting access of lipolytic enzymes to the fat substrate.

CONCLUSIONS

Heat treatment of RM had a significant impact on the diversity and numbers of microflora in the resultant Cheddar cheeses. Milk heat treatment had no effect on pH 4.6-SN or 5% PTA-SN formation; however, heat treatment reduced the levels of FAA during ripening, indicating that the indigenous proteolytic enzymes or proteases from the RM microflora influenced FAA production. Lipoprotein lipase activity in the RM was partially inactivated by thermization and extensively or wholly inactivated by pasteurization. Lipoprotein lipase was active in the vat and over the ripening period in RM and, to a lesser extent, in TM cheeses. Starter esterases were the major agents of lipolysis in PM cheeses and possibly in TM and RM cheeses. Indigenous milk microflora and NSLAB may have also contributed to lipolysis in TM and RM cheeses due to their presence at high levels from the start of ripening. The results of the study indicate that lipolytic activity of the starter esterases, indigenous microflora, and LPL appears limited due to impaired access to substrate or the physicochemical environment in cheese.

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