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Starter Bacteria Are the Prime Agents of Lipolysis in Cheddar Cheese

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To assess the contribution of starter lactic acid bacteria (LAB) to lipolysis in Cheddar cheese, the evolution of free fatty acids (FFAs) was monitored in Cheddar cheeses manufactured from pasteurized milks with or without starter. Starter-free cheeses were acidified by a combination of lactic acid and glucono-δ-lactone. Starter cultures were found to actively produce FFAs in the cheese vat, and mean levels of FFAs were significantly higher in starter cheeses over ripening. The contribution of nonstarter LAB toward lipolysis appears minimal, especially in starter-acidified cheeses. It is postulated that the moderate increases in FFAs in Cheddar cheese are primarily due to lack of access of esterase of LAB to suitable lipid substrate. The results of this study indicate that starter esterases are the primary contributors to lipolysis in Cheddar cheese made from good quality pasteurized milk.

KEYWORDS: Cheddar; lipolysis; starter-free cheese; glucono-δ-lactone

INTRODUCTION

The extent of lipolysis in Cheddar cheese is moderate as compared to many other cheese varieties (*1*, *2*); however, lipolysis is thought to play an increasingly important role in flavor development (*3*). Wijesundera and Drury (*4*) suggested that milk fat is not only a solvent for flavor compounds but also a key source of Cheddar flavor compounds.

As pasteurization appears to completely inactivate indigenous lipoprotein lipase (*5*), it is thought that starter lactic acid bacteria (LAB) or the adventitious microflora are the main source of lipolytic agents in pasteurized Cheddar cheese (*1*, *6*, *7*). A useful method to assess the biochemical contribution of starter cultures in Cheddar cheese is to compare cheeses acidified using a starter culture, with starter-free cheeses acidified using glucono-δ-lactone (GDL). In milk, GDL slowly breaks down to gluconic acid, reducing the pH, and has been used to mimic the acidification profile of starter LAB in standard cheese production (*8*−*15*). Despite the wealth of data generated on the role of starter proteolysis in cheese, a comprehensive study has not been undertaken using the GDL methodology to investigate the contribution of starter LAB to lipolysis in Cheddar cheese. Some earlier studies by Reiter et al. (*8*, *9*) used a GDL method described by Mabbit et al. (*16*) to investigate the contribution of starter LAB to lipolysis in Cheddar type cheese and indicated a contribution by starter LAB. O'Keeffe et al. (*17*) used lactic acid (LA) and GDL to better mimic the Cheddar cheese-making process, and was used in this study to investigate lipolysis. This study was undertaken to further elucidate the role of starter LAB in lipolysis of Cheddar cheese produced from pasteurized, good quality milk during production and over ripening, using up to date techniques to measure lipolysis.

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MATERIALS AND METHODS

Starter Cultures Used for Cheesemaking. *Lactococcus lactis* ssp. *lactis* 303 (303) was obtained from Chr. Hansen's Ireland Ltd. (Little Island, County Cork, Ireland), and *Lactobacillus helveticus* DPC4571 (4571) was obtained from the culture collection of Moorepark Food Research Centre (Teagasc, Fermoy, County Cork). The cultures were maintained in 10% (wt/vol) reconstituted skim milk (RSM; Golden Vale Food Products Ltd., Cork, Ireland) at −80 °C. Prior to cheesemaking, 303 and 4571 cultures were grown overnight at 23 and 37 °C, respectively, in heat-treated (95 °C for 30 min) 10% (wt/vol) RSM.

Cheese Manufacture. Cheddar cheeses were manufactured in 500 L vats on three separate occasions using early lactation pasteurized milk (72 °C × 15 s) obtained from a spring-calving Friesian herd at the Dairy Production Centre, Moorepark, standardized to a protein to fat ratio of 0.9−1.0. Three vats of cheese were made on each occasion, two of which were starter-acidified using *L. lactis* 303 (inoculum, 1.5 wt/vol) or *L. helveticus* 4571 (inoculum, 1.8 wt %/vol) and the other a starter-free cheese acidified by a combination of direct addition of LA (Galactic, Brussels, Belgium) and GDL (Pfizer Chemical Crop, Ringaskiddy, County Cork, Ireland). Rennet (Chr. Hansen's Laboratory Ltd.) was added to each vat (18 mL/100 L milk). Conventional cheese-making methods were used to manufacture the two starter-acidified cheeses (curd was cooked at 38.5 °C, pitched at pH 6.15, milled at pH 5.35, and salted at 2.7 wt %/wt). The method described by O'Keeffe et al. (*17*) was used to produce starter-free cheeses (LA−GDL) as follows:

(i) Before renneting, the milk pH was adjusted with 6% (vol/wt) LA (∼0.9 kg) to the same value as milk containing starter culture 303.

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(The acid profile development of 4571 cheeses was not followed as 4571 has poor acidification properties, which result in longer manufacturing times.) (ii) During cooking, the pH of the curd/whey was adjusted using 6% (vol/wt) LA (~2.45 kg) to mimic the pH values in cheeses made with 303. (iii) After whey drainage, 4% (wt/wt) GDL was added to the curd and mixed thoroughly. (iv) A further 2.3% (wt/wt) GDL was added at salting.

While not identical, the pH profile of 303 cheeses and that of LA−GDL cheeses followed much the same trend. Precautions taken to reduce microbial contamination when making LA−GDL cheeses included the following: filter sterilization of rennet and LA and heat treatment of diluting water to 121 °C for 15 min. All cheeses were ripened at 8 °C for 168 days and sampled at 14 days of ripening for compositional analysis; all other analyses were carried out at days 1, 14, 28, 56, 112, and 168.

Milk Quality. Coliforms in raw and pasteurized milk were enumerated on violet red bile agar (Oxoid, Basingtoke, United Kingdom), incubated at 30 °C for 24 h; total bacterial count (TBC) on milk plate count agar (Oxoid) incubated at 30 °C for 72 h; psychotrophic bacteria on milk plate agar (Oxoid) incubated at 8 °C for 10 days; enterococci on kanamycin esculin azide agar (Merck, Darmstadt, Germany) incubated at 37 °C for 24 h; and nonstarter LAB (NSLAB) incubated anaerobically on LBS agar (*18*) at 30 °C for 5 days. Staphylococci were enumerated on baird parker agar (Merck) with Egg Yolk Tellurite (Merck) supplement and incubated at 37 °C for 48 h. Lipolytic bacteria were estimated on tributyrin, triolein, and butterfat agars (Merck) incubated at 8 and 30 °C for 10 and 3 days, respectively. The somatic cell count (SCC) of the raw milk was measured on a Bentley Somacount 300 (Bentley Instrument Inc., MN).

Lipolytic Activity in Raw and Pasteurized Cheese Milks. Individual free fatty acids (FFAs) ($C_{4:0}$−$C_{18:1}$) were quantified in asceptically drawn raw or pasteurized cheese milks with 0.05% sodium azide added before and after incubation with shaking at 37 °C for 4 days by gas chromatography−flame-ionized detection (GC-FID) according to Hickey et al. (*3*).

Cheese Composition. Grated cheese samples were analyzed at 14 days in duplicate for moisture, fat, salt, and total nitrogen using standard IDF methods and for pH using the British Standard method according to Hickey et al. (*3*).

Microbiological Analysis of Cheeses. *L. Lactis* 303 was enumerated on LM17 agar (19) after 3 days at 30 °C and *Lb. helveticus* 4571 was enumerated on MRS PH5-4 agar after 3 days incubated anaerobically at 37 °C. NSLAB, coliforms, enterococci, staphylococci, TBC, and lipolytic bacteria (incubated at 30 °C for 3 days only) were enumerated over ripening.

Starter Autolysis in Cheese. Cheese juice was extracted from starter acidified cheeses as described by Wilkinson et al. (*20*) and assayed immediately for lactate dehydrogenase (LDH) activity by a modification of the method of Wittenberger and Angelo (*21*). The activity was determined by a decrease in absorbance at 340 nm (Spectronic Genesys 5 spectrophotometer, Milton Roy Company, Rochester, NY) resulting from the pyruvate (Sigma-Aldrich, Dublin 24, Ireland) dependent oxidation of nicotinamide adenine dinucelotide (NADH) (Sigma-Aldrich) in the presence (for 303) or absence of fructose-1,6-bis-phosphate (for 4571). Results are expressed as LDH units, where one unit is defined as the amount of enzyme that catalyzes the oxidation of 1 μM NADH/min/mL of cheese juice.

Assessment of Proteolysis in Cheese during Ripening. Levels of nitrogen soluble at pH 4.6 (pH 4.6-SN), 5% (w/w) phosphotungstic acid-soluble nitrogen (5% PTA-SN), and individual free amino acids (FAA) were determined on 12% trichloroacetic according to Hickey et al. (*3*).

Assessment of Lipolysis in Cheese during Ripening. Individual FFAs ($C_{4:0}$−$C_{18:1}$) in cheese were quantified by GC-FID according to Hickey et al. (*3*).

Statistical Analysis. A randomized complete block design, which incorporated the treatments, and three blocks (replicate trials) were used for analysis of the response variables relating to FFA composition of cheese milks, cheese composition, and FFA composition of cheese milks and cheeses at 1 day (**Tables 1**, **2**, and **4**). Analysis of variance (ANOVA) was carried out using the general linear model (GLM) procedure of SAS (*22*) where 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, i.e., LDH activity, pH4.6-SN, 5% PTA-SN, and concentrations of FAA and FFA. ANOVA for the split plot was carried out using the GLM procedure of SAS (*22*). Statistically significant differences ($P < 0.05$) between the different treatments were determined by Fisher's least significant differences.

Table 1. FFA Composition of Raw and Pasteurized Cheese Milks before and after Incubation at 37 °C for 4 Days[a]

FFA (mg/kg milk)	raw milk[b] 0 h	raw milk[b] 4 days	pasteurized milk[b] 0 h	pasteurized milk[b] 4 days
$C_{4:0}$	4 ± 1 d	270 ± 2 c	3 ± 0 c	4 ±2 c
$C_{6:0}$	3 ± 1 d	144 ± 7 c	2 ± 1 c	3 ± 1 c
$C_{8:0}$	3 ± 1 d	130 ± 50 c	3 ± 0 c	3 ± 1 c
$C_{10:0}$	5 ± 1 d	260 ± 50 c	5 ± 1c	5 ± 1 c
$C_{12:0}$	5 ± 1 d	244 ± 125 c	5 ± 1 c	9 ± 3 c
$C_{14:0}$	8 ± 2 d	516 ± 255 c	8 ± 0 c	12 ± 4 c
$C_{16:0}$	38 ± 4 d	1011 ± 515 c	41 ± 7 c	43 ± 12 c
$C_{18:0}$	3 ± 3 d	451 ± 179 c	2 ± 1 c	12 ± 6 c
$C_{18:1}$	21 ± 2 d	567 ± 161 c	18 ± 1 c	19 ± 9 c
total ($\sum C_{4:0}$−$C_{18:1}$)	90 ± 12 d	3437 ± 1361 c	88 ± 6 c	110 ± 18 c

[a] Data presented are the means ± SD of three replicate trials. [b] Values within rows not sharing a common letter (c, d) differ significantly; P <0.05.

RESULTS AND DISCUSSION

Bacteriological Quality of the Raw and Pasteurized Milk. The microbial flora of raw milk were comprised of a TBC ~10^4 cfu/mL, psychrotrophic bacterial count ~10^4 cfu/mL, coliforms ~10^2 cfu/mL, NSLAB ~10^2 cfu/mL, enterococci at ~10^4 cfu/mL, staphylococci at ~10^2 cfu/L, and lipolytic bacteria (on tributryin, triolien, and butterfat agar incubated at 30 °C only) ~10^3 cfu/mL (data not shown). SCCs were at 1.8×10^5 cells/mL. Pasteurization reduced the TBC to ~10^1 cfu/mL and eliminated all other bacteria. Bacterial counts and SCC in the raw and pasteurized milks were within normal ranges for Cheddar cheese manufacture (European Union, Council Directive 92/46/EEC, no. L268/1).

FFA Profile and Lipolytic Activity in Raw and Pasteurized Cheese Milks. Significant differences were not observed in the levels of individual and total FFA (TFFA) between raw and pasteurized cheese milks prior to incubation. After incubation, TFFAs in raw cheese milks increased ($P < 0.05$) 45-fold (**Table 1**). However, no significant increases were observed in TFFAs of pasteurized cheese milks, indicating inactivation of lipoprotein lipase. Slight increases in the levels of FFA in the pasteurized milk postincubation were most likely due to physical release by agitation during incubation.

Cheese Composition. The chemical composition of cheeses at 14 days is shown in **Table 2**. While most of the cheeses were within the normal range for good quality Cheddar, fat in dry matter levels (FDM) in LA−GDL and 303 cheeses were marginally below a recommended limit of 50% (w/w) prescribed by Gilles and Lawrence (*23*). Significant differences ($P < 0.05$) in moisture content were observed between cheeses (LA−GDL > 303 > 4571) and most likely are due to differences in the rates of acidification of the curds during manufacture, as reflected by significant differences in cheese pH (*24*, *25*). Similar to Lane and Fox (*13*) and Lynch et al. (*15*), it was difficult to control the pH of LA−GDL cheeses due to the difficulty in estimating the level of GDL required for cheesemaking. Levels

Table 2. Composition of Cheddar Cheeses Acidified with LA–GDL, *L. lactis* ssp. *lactis* 303 (303) and *L. helveticus* DPC4571 (4571)[a]

composition	LA–GDL	303	4571	SED[b]
moisture (g/100 g)	38.3 e	36.2 d	33.2 c	0.35
fat (g/100 g)	30.0 e	31.7 d	33.8 c	0.16
protein (g/100 g)	24.4 e	26.2 d	27.2 c	0.30
salt (g/100 g)	1.5 c	1.4 c	1.7 c	0.11
S/M (g/100 g)[b]	4.0 d	4.0 d	5.3 c	0.34
FDM (g/100 g)[b]	48.7 d	49.7 c	50.6 c	0.37
MNFS (g/100 g)[b]	54.8 c	53.1 d	50.1 e	0.52
pH	5.3 c	5.1 d	5.2 cd	0.06

[a] Data presented are the means of three replicate trials. [b] SED, standard error of difference. Values within rows not sharing a common letter (c–e) differ significantly; $P < 0.05$.

of fat, protein, and moisture in nonfat substance (MNFS) differed significantly ($P < 0.05$) between cheeses arising from significant ($P < 0.05$) variations in moisture levels. The lower fat and protein levels in LA−GDL cheeses may be due to added GDL and to the retention of lactose, both of which can increase the water-holding capacity (*12*). Significantly higher ($P < 0.05$) salt in moisture (S/M) levels of 4571 cheeses reflect the low moisture contents of these cheeses. The lower moisture content of 4571 cheeses is primarily due to its poor acidification properties, which increase manufacturing time.

Microflora of the Cheeses. The microflora of the various cheeses over ripening are shown in **Table 3**. In agreement with previous studies (*3*, *26*, *27*), populations of 303 starter were $\sim10^9$ cfu/g at 1 day in cheese and remained at this level during the first 56 days of ripening and populations of 4571 starter decreased from $\sim10^7$ cfu/g at 1 day to $\sim10^3$ cfu/g at 56 days. NSLAB increased from $<10^1$ cfu/g at 1 day to $\sim10^6$ cfu/g at 168 days in cheeses made with strain 303 or in LA−GDL cheeses. In agreement with Hannon et al. (*27*), NSLAB were markedly lower in cheeses made with strain 4571 during ripening and reached $\sim10^4$ cfu/g at 168 days.

The low levels of coliforms detected in 4571 and 303 cheeses at the start of ripening were absent by 14 and 28 days, respectively. Enterococci or staphylcocci were not present in these cheeses. Despite precautions to reduce microbial contamination during the manufacture of LA−GDL cheeses, TBCs were at $\sim10^8$ cfu/g, coliforms at $\sim10^5$ cfu/g, enterococci at $\sim10^5$ cfu/g, and staphylococci at $\sim10^5$ cfu/g (no coagulase positive staphylococci were detected) during ripening (**Table 3**). The absence of starter LAB, higher levels of lactose, in addition to the higher pH, lower S/M, and higher MNFS of LA−GDL cheeses may have provided a more favorable environment for growth of adventitious microorganisms (*15*, *28*).

Throughout ripening, low levels of lipolytic bacteria were detected on tributyrin agar in 303 and 4571 cheeses with no growth detected on triolein or butterfat agar. On average, in LA−GDL cheeses, lipolytic bacteria were detected on tributryin, triolein, and butterfat agar at $\sim10^4$ to $\sim10^5$ cfu/g. However, in one of the replicate LA−GDL trials, lipolytic bacteria were not detected on either triolein or butterfat agars. Colonies randomly isolated from tributryin/triolein agar plates were mostly Gram-positive catalase positive cocci; however, in one of the replicate LA−GDL cheese trials, Gram-negative rods were also isolated. Gilles (*29*) found Gram-positive, catalase positive cocci isolated from Cheddar were weakly lipolytic with the greater proportion belonging to the genus *Staphylococcus*.

Starter Autolysis. Starter autolysis was monitored in extracted cheese juice during the early stages of ripening when starter LAB were the predominant population (data not shown).

Table 3. Microflora of Cheddar Cheeses Acidified with LA–GDL, *L. lactis* ssp. *lactis* 303 (303) and *L. helveticus* DPC4571 (4571) during Ripening[a]

population enumerated	ripening time (days)	cheeses (cfu/g cheese)[b,c]		
		LA–GDL	303	4571
starter	1		2.6×10^9	7.0×10^7
	14		2.2×10^9	5.8×10^7
	28		6.7×10^9	2.8×10^6
	56		2.8×10^9	4.2×10^3
NSLAB	1	2.8×10^1	>10	ND
	14	1.3×10^3	2.0×10^2	>10
	28	5.2×10^3	1.9×10^2	8.3×10^1
	56	4.2×10^4	9.1×10^2	1.7×10^2
	112	9.6×10^5	4.1×10^4	3.7×10^2
	168	9.6×10^5	7.8×10^6	4.7×10^4
coliforms	1	5.1×10^5	>10	2.3×10^1
	14	2.2×10^6	>10	ND
	28	4.2×10^4	ND	ND
	56	5.2×10^5	ND	ND
	112	7.1×10^3	ND	ND
	168	1.9×10^4	ND	ND
enterococci	1	2.8×10^3	ND	>10
	14	6.8×10^4	ND	ND
	28	1.2×10^4	ND	ND
	56	2.2×10^5	ND	ND
	112	2.2×10^5	ND	ND
	168	3.7×10^5	ND	ND
staphylococci	1	3.2×10^4	ND	ND
	14	1.8×10^6	ND	ND
	28	1.6×10^5	ND	ND
	56	7.6×10^4	ND	ND
	112	3.3×10^5	ND	ND
	168	1.0×10^5	ND	ND
tributryin hydrolyzing	1	5.6×10^4	3.4×10^1	>10
	14	7.0×10^5	1.3×10^3	1.0×10^1
	28	5.0×10^5	>10	6.0×10^1
	56	6.4×10^4	3.1×10^1	ND
	112	8.2×10^4	3.7×10^1	ND
	168	9.3×10^4	1.3×10^2	>10
triolein hydrolyzing	1	2.1×10^4	ND	ND
	14	5.9×10^5	ND	ND
	28	4.7×10^5	ND	ND
	56	1.7×10^5	ND	ND
	112	3.2×10^5	ND	ND
	168	1.0×10^5	ND	ND
butterfat hydrolyzing	1	nd	ND	ND
	14	1.5×10^5	ND	ND
	28	4.2×10^4	ND	ND
	56	7.7×10^4	ND	ND
	112	ND	ND	ND
	168	ND	ND	ND
total bacterial count	1	3.0×10^8	4.5×10^8	2.9×10^7
	14	1.4×10^8	5.9×10^8	4.6×10^7
	28	2.0×10^7	4.5×10^9	1.4×10^6
	56	1.2×10^8	1.2×10^9	2.3×10^5
	112	1.2×10^8	7.0×10^9	1.3×10^5
	168	2.0×10^7	6.1×10^8	2.8×10^4

[a] Data presented are the means of three replicate trials. [b] Blank cell, not determined. [c] ND, not detected in 1 g of cheese.

In agreement with other authors, low levels of LDH activity were detected in 303 cheeses (*26*) indicating poor autolysis while high levels of LDH were detected in 4571 cheeses (*27*, *30*) indicating extensive autolysis. LDH activity was not monitored in LA−GDL cheeses as these cheeses had a variable microflora and the origin of any LDH activity would have been unknown.

Proteolysis. Indices of proteolysis pH 4.6-SN, 5% PTA-SN, and FAA are shown in **Figure 1**. ANOVA of the pH 4.6-SN revealed that significant differences ($P < 0.05$) existed due to ripening time (df = 5, F = 138, and $P < 0.000$). However, significant differences were not observed for the type of acidification indicating that the coagulant and indigenous milk

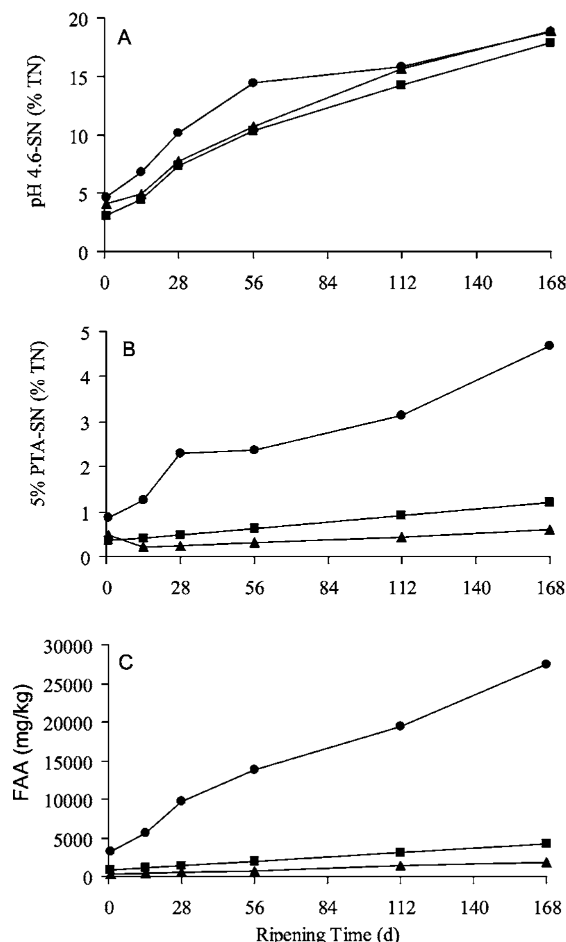

Figure 1. Proteolytic indices (**A**) pH 4.6-SN, (**B**) 5% PTA-SN, and (**C**) FAAs in Cheddar cheeses acidified with LA and GDL (▲), *L. lactis* ssp. *lactis* 303 (■) and *L. helveticus* DPC4571 (●) during ripening. Values presented are the means of three replicate trials.

proteinase (plasmin) were primarily responsible for the production of the pH 4.6-SN throughout ripening in agreement with previous studies (*9*−*12*, *14*, *15*).

ANOVA of the 5% PTA and FAA revealed significant differences ($P < 0.05$) existed due to type of acidification (5% PTA: df = 2, $F = 855$, and $P < 0.000$; FAA: df = 2, $F = 846$, and $P < 0.000$), ripening time (5% PTA: df = 5, $F = 3$, and $P < 0.000$; FAA: df = 5, $F = 145$, and $P < 0.000$), and the interaction of type of acidification and ripening time (5% PTA: df = 10, $F = 1$, and $P < 0.000$; FAA: df = 10, $F = 76$, and $P < 0.000$). Mean levels of 5% PTA-SN, which measure peptides and FAAs with molecular masses of <600 Da (*31*) and total FAAs decreased in the order 4571 ≫ 303 > GDL (**Figure 1**). In agreement with previous studies (*13*−*15*), peptidases from starter LAB appear to be the primary contributors to the production of low molecular mass peptides and FAAs. Extensive secondary proteolysis was evident in cheeses manufactured with 4571, which is in agreement with Hannon et al. (*27*). Lower levels of secondary proteolysis in cheeses produced with 303 are also in agreement with O'Donovan et al. (*26*) as this strain does not lyse readily nor release peptidase in cheese (*32*). The limited formation of small peptides and FAA in the LA−GDL cheeses is in agreement with previous studies (*10*−*15*) and may be due to the action of plasmin, NSLAB, and/or adventitious microorganisms. Higher MNFS in LA−GDL cheeses in addition to higher pH may also have provided more suitable conditions for microbial and enzymatic activity in these cheeses (*24*, *28*).

Table 4. Concentrations of SCFFA ($C_{4:0}$–$C_{8:0}$), MCFFA ($C_{10:0}$–$C_{14:0}$), and LCFFA ($C_{16:0}$–$C_{18:1}$) in Cheese Milks and Cheddar Cheeses Acidified with LA–GDL, *L. lactis* ssp. *lactis* 303 (303) and *L. helveticus* DPC4571 (4571) at 1 Day[a]

mg/kg fat	cheese milk	LA–GDL	303	4571	SED[b]
SCFFA	261 d	38 f	59 e	36 f	7
MCFFA	522 d	222 f	475 d	359 e	28
LCFFA	1846 d	1310 e	1293 e	1449 e	68
total FFA	2629 d	1570 e	1827 e	1844 e	103

[a] Data presented are the means of three replicate trials. [b] SED, standard deviation of difference. Values within rows not sharing a common letter (d–f) differ significantly; P <0.05.

Lipolysis. It was evident from the LA−GDL cheeses that ~40% of the TFFAs present in the cheese milks were lost during the cheese-making process (**Table 4**). This is in agreement with Nelson and Barbano (*33*), who recently reported that 40−45% of FFAs are lost in the whey during Cheddar cheese manufacture. Losses were lower in the cheeses produced with the starter cultures 303 and 4571 (~30%) than in LA−GDL cheeses (~40%). Short ($C_{4:0}$−$C_{8:0}$; SCFFA), medium ($C_{10:0}$−$C_{14:0}$; MCFFA), and long ($C_{16:0}$−$C_{18:1}$; LCFFA) chain FFA levels in cheese milks and cheeses at 1 day are shown in **Table 4**. Approximately 85 and 57% of SCFFA and MCFFA were lost in the whey during manufacture of LA−GDL cheese as opposed to 29% of LCFFA. The partitioning of SCFFA and MCFFA with the whey during drainage was most likely due to their higher water solubility, while losses of some MCFFA and LCFFA may be related to the association of these apolar compounds with β-lactoglobulin and/or serum albumin (*34*). Similar levels of SCFFA were lost in 303, 4571, and LA−GDL cheeses, but significantly lower levels of MCFFA were lost in starter-acidified cheeses. Differences in losses of MCFFA between the starter cheeses (303 and 4571) and the LA−GDL cheeses were most likely due to generation of MCFFA by the starters in the vat during manufacture (**Table 4**). SCFFAs were also likely to have been produced in the vat in the starter cheeses, but as MCFFAs are less water-soluble than SCFFA more may have partitioned with the curd rather than the whey. Losses of LCFFA in all cheeses were similar.

Similar to Reiter et al. (*8*, *9*), significantly lower ($P < 0.05$) levels of TFFAs were observed in LA−GDL cheeses as compared with 303 and 4571 cheeses at 1 day, indicating that the starters (303 and 4571) actively liberate FFA in the vat (**Table 5**). Although significant differences in TFFA levels were not observed between 303 and 4571 cheeses at 1 day, TFFA levels were numerically higher in 4571 cheeses. The 4571 cheese may release more FFA in the vat than 303, due to its higher autolytic properties, as esterases in LAB appear to be exclusively intracellular (*1*) or due to a greater lipolytic activity and/or to an extended manufacture time in the vat. It is also likely that if 4571 produced higher levels of SCFFA in the vat, they would have been predominantly lost with the whey due to their solubility. It was not possible to quantify losses of FFA in the whey directly due to their low concentration. Esterases of starter cultures may be active in the vat once lysis has occurred and may reach maximum activity during the scald (32−38 °C), as these temperatures are closer to their optimum (*1*) and closer to the phase transition temperature of the components of the milk fat globule membrane (*35*), thereby providing greater access to suitable lipid substrates.

ANOVA of the TFFA revealed that significant differences ($P < 0.05$) existed due to type of acidification (df = 2, $F = 110$, and $P < 0.000$), ripening time (df = 5, $F = 62$, and $P <$

Table 5. Concentrations of FFA ($C_{4:0}$–$C_{18:1}$) and ANOVA of Cheddar Cheeses Acidified with LA–GDL, *L. lactis* ssp. *lactis* 303 (303) and *L. helvticus* DPC4571 (4571) during Ripening[a]

cheese	ripening time	FFA									
		$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	total
GDL	1	5 ± 8	1 ± 1	6 ± 3	5 ± 7	15 ± 9	46 ± 22	128 ± 42	70 ± 18	194 ± 41	471 ± 50
	14	5 ± 7	2 ± 1	7 ± 3	11 ± 9	20 ± 14	55 ± 11	148 ± 24	68 ± 24	184 ± 21	499 ± 49
	28	5 ± 6	3 ± 0	7 ± 3	12 ± 11	22 ± 17	56 ± 8	156 ± 25	67 ± 19	192 ± 36	520 ± 65
	56	6 ± 7	2 ± 1	7 ± 3	13 ± 10	23 ± 17	59 ± 6	157 ± 30	68 ± 21	195 ± 33	531 ± 64
	112	9 ± 6	5 ± 2	9 ± 3	21 ± 8	31 ± 16	65 ± 12	170 ± 40	65 ± 20	203 ± 41	578 ± 99
	168	9 ± 6	5 ± 2	10 ± 3	22 ± 9	31 ± 19	67 ± 9	174 ± 30	69 ± 21	208 ± 38	595 ± 87
303	1	11 ± 1	3 ± 0	5 ± 1	30 ± 4	47 ± 4	73 ± 2	185 ± 16	57 ± 9	168 ± 22	580 ± 40
	14	14 ± 0	3 ± 0	5 ± 1	31 ± 4	49 ± 5	83 ± 2	189 ± 18	60 ± 11	167 ± 13	601 ± 35
	28	14 ± 5	4 ± 1	8 ± 3	28 ± 10	46 ± 14	96 ± 13	201 ± 44	67 ± 24	174 ± 28	636 ± 87
	56	22 ± 3	5 ± 1	6 ± 1	35 ± 5	54 ± 5	96 ± 0	190 ± 14	59 ± 11	168 ± 15	635 ± 36
	112	30 ± 6	7 ± 5	7 ± 2	37 ± 8	59 ± 6	107 ± 7	208 ± 19	62 ± 13	189 ± 33	705 ± 66
	168	39 ± 5	10 ± 3	11 ± 4	36 ± 11	57 ± 17	118 ± 14	238 ± 42	75 ± 24	208 ± 40	791 ± 112
4571	1	0 ± 0	3 ± 2	9 ± 1	9 ± 7	18 ± 7	95 ± 4	211 ± 33	83 ± 9	196 ± 22	624 ± 70
	14	2 ± 1	3 ± 0	10 ± 1	13 ± 6	23 ± 6	105 ± 3	226 ± 29	85 ± 10	215 ± 25	682 ± 62
	28	4 ± 1	4 ± 0	10 ± 1	14 ± 5	26 ± 5	118 ± 7	236 ± 37	87 ± 7	225 ± 31	725 ± 86
	56	11 ± 2	4 ± 1	11 ± 2	16 ± 5	28 ± 6	123 ± 6	239 ± 32	89 ± 9	223 ± 32	744 ± 82
	112	22 ± 5	5 ± 2	11 ± 2	20 ± 5	33 ± 4	134 ± 6	259 ± 30	91 ± 9	239 ± 35	815 ± 87
	168	32 ± 4	7 ± 1	13 ± 2	26 ± 8	37 ± 8	144 ± 13	277 ± 43	92 ± 4	247 ± 36	874 ± 109
		ANOVA[b]									
treatment[c]		*	**	NS	**	**	**	**	*	*	***
ripening time		***	***	***	***	***	***	***	**	***	***
interaction: treatment × ripening time		***	**	NS	**	NS	***	***	NS	NS	*

[a] Numbrs represent means ± SD. [b] Significance levels: *P < 0.05; P < 0.01; ***P < 0.001; NS, not significant. [c] Treatment: acidified with LA–GDL, 303, or 4571.

0.000), and the interaction of type of acidification and ripening time (df = 10, F = 3, and P < 0.020). Levels of TFFA decreased in the order 4571 > 303 > LA−GDL (**Table 5**) over ripening. During ripening, the rate of increase of SCFFA was similar in 303 and 4571 cheeses and significantly higher (P < 0.05) than in LA−GDL cheeses. No significant differences in the rate of MCFFA generated over ripening were observed between all of the cheeses. The 4571 cheeses had significantly higher (P < 0.05) levels of LCFFA as compared to 303 and LA−GDL cheeses. The higher levels of SCFFA in starter-acidified cheeses as compared to LA−GDL cheeses over ripening may be attributed to esterase activity. To date, esterase activity has been identified in *L. lactis* (*36*−*38*) and *L. helveticus* (*39*). NSLAB also possess esterases (*40*), and it is thought that these esterases may contribute to the release of FFA (*6*, *7*). However, in 4571 cheeses, NSLAB levels were very low throughout ripening, and in 303 cheeses, NSLAB levels only reached ~10^6 cfu/g at the latter stages of ripening and, therefore, are unlikely to have contributed toward lipolysis during the early stages of ripening (*41*). Significantly higher (P < 0.05) levels of TFFA were observed in 4571 than in 303 cheeses over ripening (**Table 5**). This may be due to increased autolysis of 4571 or lipolytic activity, as a positive correlation has been suggested between autolysing starters and FFA production in Cheddar cheese during ripening (*42*). Compositional differences (lower moisture and MNFS) in 4571 cheeses as well as extensive proteolysis of the casein matrix during ripening may have led to increased fat accessibility (*43*); however, although significant compositional differences were evident, they were thought to be too small to influence fat accessibility.

In agreement with Collins et al. (*42*), mean levels of TFFA increased significantly (P < 0.001) over ripening. The increase in TFFA over ripening in 303 and 4571 cheeses was modest in comparison to other cheese varieties, such as Blue and hard Italian cheeses. Mean levels of TFFA increased by 124, 211, and 250 mg per kg cheese in LA−GDL, 303 and 4571 cheeses, respectively, highlighting a 2- and 1.7-fold respective increase in 4571 and 303 over LA−GDL cheeses. The relative small increase in lipolysis in Cheddar cheese may be due to limited access of starter esterases to suitable fat substrates in cheese curd. Carunchia Whetstine et al. (*44*) have suggested that little interaction occurs between the aqueous phase in Cheddar cheese and the liquid fat phase, as the liquid fat is surrounded by a solid fat phase. It has also been reported that triacylglycerides are partially crystallized over the early stages of semihard cheese ripening (*45*). These factors would severely limit interaction of water-soluble starter LAB esterases in the aqueous phase with their preferred substrate, i.e., mono- and diacylglycerides present in the liquid fat phase (*46*).

The significant difference between the extent of lipolysis in LA−GDL and 303 and 4571 cheeses in this study clearly highlights the major contribution of starter esterases toward lipolysis in Cheddar cheese. The contribution of NSLAB toward lipolysis appears minimal as despite higher levels of NSLAB earlier during ripening levels of TFFA were lowest in LA−GDL cheeses. In addition, the lipolysis in LA−GDL cheeses most probably also arose from a combination of esterases from enterococci, staphylococci, or other bacterial sources present in these cheeses (*29*, *47*, *48*), which would typically not be present in Cheddar produced with starter bacteria, as shown in this study.

Conclusions. This study demonstrates that starter esterases are the primary contributors to lipolysis in Cheddar cheese and that a significant level of lipolytic activity from the starter occurs in the vat during cheese manufacture. The lipolytic activity in the vat has a major impact on the level of TFFAs in the resultant cheese, despite considerable losses of water-soluble FFAs during drainage. It is postulated that esterases of starter bacteria have greater access to suitable lipid substrates in the vat than in cheese curd over ripening due to cheese-processing physicochemical conditions. In addition, it appears that the contribution of NSLAB toward lipolysis in Cheddar cheese is minimal as levels were lowest in LA−GDL cheeses, despite having the highest levels of NSLAB earlier in ripening. It also appears that the lipolytic activity in the LA−GDL cheeses may have arisen from

other contaminating bacteria that grew well in these cheeses but would not typically be present in starter-acidified Cheddar cheese.

ABBREVIATIONS USED

LAB, lactic acid bacteria; FFAs, free fatty acids; LA, lactic acid; GDL, glucono-δ-lactone; RSM, reconstituted skim milk; TBC, total bacteria count; SCC, somatic cell count; GC-FID, gas chromatography−flame-ionized detection; LDH, lactose dehydrogenase; NADH, nicotinamide adenine dinucelotide; pH 4.6-SN, pH 4.6 water-soluble nitrogen; PTA-SN, phosphotungstic acid-soluble nitrogen; FAAs, free amino acids; ANOVA, analysis of variance; GLM, general linear model; TFFA, total free fatty acid; FDM, fat in dry matter; MNFS, moisture in nonfat substances; S/M, salt in moisture; NSLAB, nonstarter lactic acid bacteria; SCFFA, short-chain free fatty acids; MCFFA, medium-chain free fatty acids; LCFFA, long-chain free fatty acids.

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