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ARTICLE

Time Course and Specificity of Lipolysis in Swiss Cheese

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Controlling lipolysis in cheese is necessary to ensure the formation of desirable flavor. To get a better understanding of the mechanism of lipolysis in Swiss cheese, cheeses were manufactured with and without (control) the addition of *Propionibacterium freudenreichii*. Products of lipolysis were quantified throughout ripening. Half of the free fatty acids (FFA) released in milk (3.66 mg/g fat), in particular the short-chain FFA, were lost in the whey during curd drainage, whereas diglycerides and monoglycerides were retained within the curd. *P. freudenreichii* was responsible for the release of most FFA during ripening (10.84 and 0.39 mg/g fat in propionibacteria-containing and control cheeses, respectively). Indices of lipolysis displayed low specificity. All types of FFA were released, but butyric and palmitic acids more significantly, which could be due to a low *sn*-1,3 regioselectivity. All glycerides were hydrolyzed in the following order: monoglycerides > diglycerides > triglycerides. The results of this study show the quantitative and qualitative contributions of the different lipolytic agents to Swiss cheese lipolysis.

KEYWORDS: Swiss cheese; lipolysis; *Propionibacterium freudenreichii*; glycerides

INTRODUCTION

Lipolysis is an essential phenomenon in the formation of cheese flavor. It consists of the hydrolysis of glycerides and results in the release of free fatty acids (FFA). Short- and medium-chain FFA (4−12 carbon atoms) contribute strongly to cheese flavor, resulting in "rancid", "cheesy", "pungent", "blue cheese", and "soapy" notes (*1*, *2*). FFA are also known to be precursors of other flavor compounds in cheese, such as esters. Levels of lipolysis are highest (> 30 mg of FFA/g of cheese, i.e., > 100 mg of FFA/g of fat) in blue cheeses, due to the high lipolytic activity of dairy molds. In contrast, levels of lipolysis are much lower in internally bacteria-ripened cheeses such as Cheddar and Swiss cheeses (0.6−1.6 and 3−4 mg of FFA/g of cheese, respectively, i.e., 2−5 and 10−13 mg of FFA/g of fat) (*2*−*6*).

A typical flavored Swiss cheese contains 10−13 mg of FFA/g of fat, whereas rancid flavor defects were observed when the concentration of FFA was about 2 times greater (*2*, *3*). Controlling the intensity of lipolysis in Swiss cheese is thus important. Swiss cheese, also called Emmental cheese, was initially manufactured in a restricted area of Switzerland. It is now manufactured in other countries and consumed internationally, with a production volume of ∼700,000 tons/year. To control the final level of FFA in cheese, better knowledge of the contribution of the different lipolytic agents in Swiss cheese and their specificity of action is necessary.

Lipolysis occurs from the initial steps of cheese manufacture, as soon as in the cheese milk and until the end of the ripening process. Its extent at each stage depends on the cheese technology employed. The enzymes involved in cheese lipolysis are milk lipoprotein lipase (LPL), microbial lipolytic enzymes, and, in some cheese varieties, exogenously added mammalian pregastric esterases. In Swiss cheese, manufactured from raw or heated milk, LPL is believed to remain at least partly active during the first steps of manufacture, but is inactivated after the "cooking" step (*7*). *Propionibacterium freudenreichii*, a species used as a ripening culture, has been shown to be a major agent in Swiss cheese lipolysis (*3*, *8*, *9*). The concentrations of FFA at the end of ripening of experimental Swiss-type cheeses were about 5 times greater in cheeses inoculated with *P. freudenreichii* than in the control (not inoculated by propionibacteria) (*8*). The majority (68%) of lipolysis occurred concomitantly with the growth of *P. freudenreichii*, during ripening in the warm room (21−24 °C) (*10*).

Hydrolysis of cheese fat, which is mainly composed of triglycerides (TG), results from the activity of the lipolytic esterases. Lipolytic esterases are esterases that are active on insoluble lipids and are commonly called lipases (*11*). They hydrolyze the ester bond between the fatty acid (FA) and the glycerol core of a glycerol ester. They display different types of specificity: the type of substrate they preferentially hydrolyze, the FA preferentially released, and the position (*sn*-1,2,3) of the ester bonds preferentially hydrolyzed on the glycerol backbone. The hydrolysis of TG results in the formation of FFA and diglycerides (DG). The DG can subsequently be further hydrolyzed, leading to the formation of FFA and monoglycerides (MG), the hydrolysis of which forms glycerol and FFA. The specificity of the lipolytic agents in cheese has only been partially described. The lipolytic enzymes of lactic acid bacteria (LAB) preferably hydrolyze MG, compared to DG, and DG compared to TG (*1*, *12*, *13*). They also preferentially release short-chain FA (C4:0, C6:0), but possess activity up to

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C12:0, even if their specificity is species- and, possibly, strain-dependent. In *P. freudenreichii*, an extracellular lipolytic enzyme that has shown activity on an emulsion of anhydrous milk fat has recently been identified (*14*). The specificity of activity of this enzyme on milk glycerides has, however, not been reported.

Lipolysis in Swiss cheese has been partially described (*2*, *3*, *8*−*10*, *15*). In most studies, however, the concentration of FFA was given for the ripened cheeses only, and neither the FA composition of the cheese fat nor the concentration of DG and MG was determined. The aim of the present study was to determine the time course and apparent specificity of the different agents of lipolysis in Swiss cheese and, more precisely, (i) to investigate the course of lipolysis, in particular during the early stages of manufacture and during the warm room ripening period, (ii) to quantify the role of LAB versus *P. freudenreichii* during the ripening period, and (iii) to shed light on the specificity of release of partial glycerides and FFA. To this end, the products of lipolysis, FFA, MG, and DG, were quantified throughout the manufacture and the ripening of experimental Swiss cheeses manufactured with and without the addition of *P. freudenreichii* as a ripening culture.

MATERIALS AND METHODS

Cheese Manufacture. Raw milk from a local herd (PCA count < 5000 colony-forming units (cfu)/mL, somatic cell count < 200,000 cells/mL) was skimmed at 50 °C and then microfiltered on a 1.4 μm gradient of porosity membrane (Pall Membralox GP, Pall France, Saint Germain en Layes, France). The fat content of cream was adjusted to 400 g/kg by the addition of microfiltered skim milk, whichg was then heat treated at 115 °C for 20 s. Microfiltered skim milk and heated cream were blended to obtain a 1.15 fat/casein ratio. Small-scale ($^1/_{100}$) experimental Swiss cheeses were manufactured from microfiltered milk according to a standardized cheesemaking process previously described (*16*, *17*). Two trials of two 800 g cheeses, one containing *P. freudenreichii* (prop-cheese) and one control cheese without *P. freudenreichii*, were manufactured at a 1 week interval, from two different batches of milks. The strain used was *P. freudenreichii* subsp. *shermanii* CIRM-BIA1^T (also referred to as CIP103027).

The lactic starters used in this study were (i) *Streptococcus thermophilus* bulk starters (PALITG ST20/87, Laboratoires Standa, Caen, France) grown on Marstar 412A (Danisco France, Dangé Saint Romain, France) and (ii) a freeze-dried mixture (Direct Vat Inoculum) of *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis* (LH100, Danisco). The curd and whey mixture was cooked at 53.5 °C for 45 min. The pressing and acidification steps were performed in thermostated ovens to mimic the thermal conditions that occur in regular size Emmental cheese.

Each cheese was brined on day 1 and then divided into one cheese block of 400 g and 8 sectors of 50 g before being wrapped under vacuum in a Cryovac BK1L film (Cryovac-Europe, F-28 Epernon, France). Cheeses were ripened at 12 °C for 10 days, then transferred to 24 °C (warm room) for 21 days, and finally maintained at 4 °C for a further 15 days. Samples were taken at nine stages of manufacture and ripening: cheese milk (cheese milk before starter and rennet addition), curd at the end of scalding (around 150 min after starter inoculation), curd 30 min after dipping, and curd at 20 h (i.e., after acidification by lactic starters), at day 10 (end of cold room), at day 17 (after 1 week in the warm room), at day 24 (after 2 weeks in the warm room), at day 31 (at the end of ripening in the warm room), and at day 45 (end of ripening). The samples were kept frozen at −76 °C until biochemical analysis.

Microbiological Aalysis. Fresh samples of cheeses (10 g) were dispersed in 90 g of a 2% sodium citrate solution, homogenized, diluted with peptone saline solution, and plated on specific media. Propionibacteria were enumerated on lithium−glycerol agar (*18*) incubated at 30 °C anaerobically for 6 days. Nonstarter LAB, which can grow in cheeses during the ripening, were enumerated on FH agar (*19*) incubated at 30 °C anaerobically for 3 days.

Compositional Analysis. Cheese milk and whey were sampled before starter inoculation and just after molding, respectively. Samples of curd were taken at the end of scalding of the curd and whey mixture, 30 min after molding, and at day 1. The fat content of milk was determined by using the Gerber acido-butyrometric method and the fat content of curd and cheese by using the Heiss butyrometric method. Samples of ripened cheeses were analyzed for moisture (oven-drying at 103 °C), for fat (Heiss method), and for pH.

Extraction of Total Lipids from Milk and Cheese for Determination of FFA, TLC, and HPLC. Total fat was extracted from milk and cheese (respectively 10 mL or 1 g) with diethyl ether/heptane (50:50, v/v), after grinding with sodium sulfate and the addition of sulfuric acid (*20*). The extractions were conducted in duplicate for each sample.

Determination of FFA. FFA (C4:0−C18:3) were analyzed by gas chromatography (GC) according to the method of De Jong and Badings (*20*), as previously reported (*10*). Briefly, FFA were isolated from total lipids using an aminopropyl column and separated on a BP21 (SGE, Ringwood, VIC, Australia) capillary column, 25 m × 0.53 mm × 0.5 μm film thickness, under the following conditions: on-column injection at 65 °C; carrier gas, hydrogen, 31 kPA; temperature program, heating rate, 10 °C/min from 65 °C to 240 °C, maintained for 10 min; flame ionization detector operated at 240 °C. The main isomer of C18:2 was *cis*-9,12-C18:2. This compound is further referred to as C18:2.

Analysis of Lipid Classes by TLC. Analysis of the evolution of lipid classes during cheese ripening was performed by TLC. Total lipid extracts corresponding to the different cheese ripening stages were dissolved in chloroform (10 mg/mL) and spotted on a silica gel plate (100 × 200 mm, 0.25 mm, Si G60, Merck) using an automatic TLC sampler (ATS3, Muttenz, Switzerland). The plate was then developed in hexane/diethyl ether/acetic acid (70:30:2 v/v/v). The spots corresponding to the different classes of lipids were visualized by heating the plates (20 min, 150 °C), after staining by immersion in a copper sulfate II/orthophosphoric acid solution.

Quantification of MG and DG by HPLC. The MG and DG were quantified by high-pressure liquid chromatography (HPLC, Agilent HP 1100, Agilent, Massy, France) with three solvent lines, a degasser, and a ternary pump, combined with an evaporative light scattering detector (ELSD, PL-ELS1000, Polymer Laboratories, Marseille, France). Data from the ELSD were acquired by HPChem software (Agilent). A thermoregulated (40 °C) silica column, 150 × 3 mm with 3 μm particle diameter (AIT, Houilles, France), and a precolumn of silica with the same packing and internal diameter were used. Dried and filtered compressed air was used as the nebulizing gas at a flow rate of 1.7 L/min and a temperature of 50 °C. The evaporating temperature was 85 °C. The elution program used was as follows: (i) t0 min, chloroform/hexane/methanol (v/v/v) 100:0:0; (ii) t7 min, 60:40:0; (iii) t10 min, 0:100:0; (iv) t12 min, 100:0:0; (v) t15 min, 90:0:10; (vi) t20 min, 90:0:10; and (vii) t35 min, 100:0:0. The flow rate was maintained at 0.5 mL/min. The injection volume was 10 μL per sample. The extracted lipids were weighed (30 mg) and dissolved in 1 mL of chloroform/hexane (90:10, v/v) and transferred into capped test tubes for HPLC analysis. Each sample was injected at least three times. Quantification of the MG and DG was performed using calibration curves of pure standards of mono- (31-1810-9, Larodan, > 99%) and diolein (32-1812-0, Larodan, > 99%).

Determination of Total Fatty Acids. Total lipids were extracted from 1 g of cheese with a mixture of ethyl alcohol, diethyl ether, and petroleum ether as previously described (*21*). Methyl esters of FA were prepared from total lipids as previously described (*22*). FA methyl esters were analyzed by GC (Agilent 7890A, Agilent, Massy, France) equipped with a flame ionization detector, a programmed temperature injector, and two capillary columns mounted in series (50 m × 0.32 mm; film thickness = 0.25 μm each one) coated with 70% cyanopropyl polysilylphenylene−siloxane (BPX-70, SGE). Chromatographic conditions were as follows: initial temperature of on-column injection (1 μL), 40 °C for 0.2 min; injector temperature program, increase to 200 °C at a rate of 200 °C/min, an isotherm at 200 °C for 6 min, and a decrease in temperature to 40 °C at a rate of 200 °C/min; detector temperature, 250 °C; carrier gas, hydrogen, at 138 kPa. The oven temperature was programmed as follows: 50 °C for 10 min followed by an increase to 175 °C at a rate of 10 °C/min and maintained at this temperature for 27 min; then the temperature was increased to 215 °C at a rate of 4 °C/min and maintained for a further 16 min. Total analysis time was 75.5 min. Each cheese sample was extracted in duplicate, methylated, and analyzed in duplicate by GC.

Statistical Analyses. Average values of replicate analyses of each compound in duplicate cheeses were used for statistical analysis. One-way

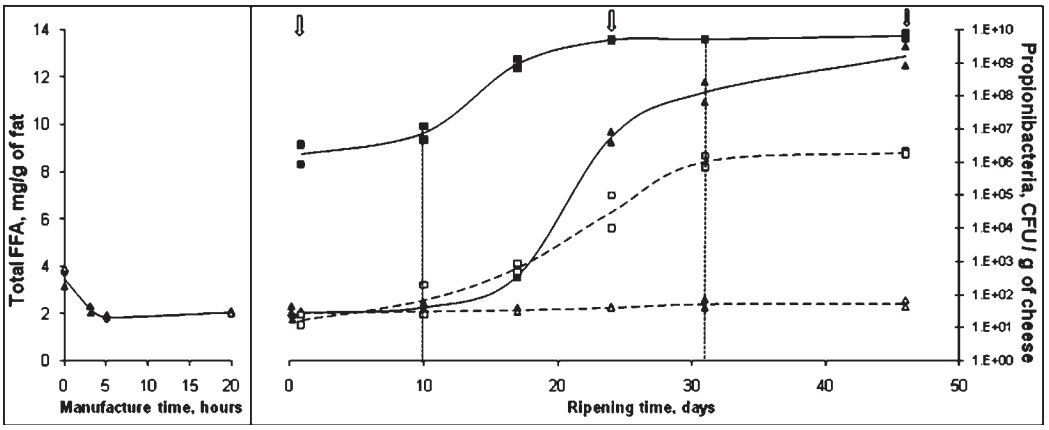

Figure 1. Propionibacteria growth curves (squares) and time course of release of total free fatty acids (FFA) (triangles) during the manufacture and ripening of Swiss-type cheese manufactured with (solid symbols) or without (open symbols) the addition of *P. freudenreichii* as ripening culture. The periods of ripening in the different rooms at 12, 24, and 4 °C are indicated by vertical dotted bars. Arrows indicate the stage of parallel TLC analyses.

analyses of variance were performed using R (http://www.R-project.org) to determine (i) the effect of the stage of ripening on the concentrations of FFA, on their relative proportions, and on their percentage of release, and (ii) the effect of the fatty acid on the loss during curd drainage. Differences between the treatment means were compared at the 5% level of significance using Fisher's least significance difference (LSD) test.

RESULTS

Cheese Composition. The gross composition of the ripened cheeses (62.4 ± 0.7% total solids, 47.6 ± 0.5% fat in dry matter, and 53.5 ± 0.5% moisture in the nonfat substance) was consistent with values expected for this type of mini-cheese (*8*, *16*) and did not significantly differ between the two batches of milk used (data not shown).

Nonstarter LAB populations varied from 10^2-10^3 cfu/g in the curd to 10^7-10^8 cfu/g in the ripened cheeses and were not significantly influenced by the batch of cheese or the presence of *P. freudenreichii* (data not shown).

Changes in FFA Concentrations during the Manufacture and Ripening of Swiss-Type Cheeses. The growth curves of propionibacteria and the time course of total FFA formation during the manufacture and ripening of Swiss-type cheeses inoculated or not with *P. freudenreichii* as a ripening culture are shown in **Figure 1**. In all cheeses, the growth of propionibacteria occurred predominantly during the warm room ripening period, as expected. In the control cheeses without added propionibacteria, the populations of indigenous propionibacteria reached 2×10^6 cfu/g at the end of ripening, that is, a value 3000-fold lower than in prop-cheeses. In the latter cheeses, the number of propionibacteria increased from 2×10^6 cfu/g in the curd to 6×10^9 cfu/g at the end of warm room period (day 31) and was stable during the subsequent cold ripening stage (**Figure 1**).

Fifteen FFA were separated and identified by GC. Only the 10 main FFA, which constituted >96% (w/w) of these 15 FA, were used for calculations. Apart from these 10 main FFA (**Table 1**), low amounts of heptanoic acid (C7:0), pelargonic acid (C9:0), undecylic acid (C11:0), palmitoleic acid (C16:1), and linolenic acid (C18:3) were also identified. The average initial concentrations of FFA in the milks used for the manufacture of the cheeses were 3.66 ± 0.35 mg/g of fat. This initial concentration markedly decreased during the draining of the curd to reach 2.04 ± 0.05 mg/g of fat in curd, due to a loss of the FFA in whey. At the earliest stages of ripening (from 20 h to curd to day 10), the concentrations of total FFA did not significantly differ between the control cheeses and the cheeses with added propionibacteria. From day 17, the amounts of FFA differed in the two types of cheeses. The concentrations of total FFA showed a very small increase in the control cheeses over the whole ripening period, whereas in prop-cheeses, the concentration of FFA increased sharply (0.39 ± 0.15 vs 10.84 ± 0.52 mg/g of fat from curd to day 46). The increase of total FFA concentration was concomitant with the growth of *P. freudenreichii* and began when the cheeses were transferred to the warm room (at day 10). More precisely, the majority of the FFA were released during the second week in the warm room, after *P. freudenreichii* reached populations above 1×10^9 cfu/g (increase from 3.60 ± 0.09 to 9.46 ± 0.33 per gram of fat). After this stage, that is, during the stationary phase of growth of *P. freudenreichii* (in the warm room and during further storage of cheese at 4 °C), lipolysis continued at a lower rate (**Figure 1**).

Changes in Individual FA Concentrations. The concentrations of total and individual FFA, expressed as milligrams per gram of fat, are listed in **Table 1** so as to allow comparison with most published studies. In addition, to illustrate the distribution of FFA, the relative concentrations, expressed as percent mole per mole of total FFA, are shown in **Figure 2**. The esterified FA composition of cheese fat was used as a reference to estimate the specificity of FFA release. It was determined in ripened cheeses only, because it had previously been shown that the distribution of esterified FA did not significantly differ in a cheese milk and in the Swiss cheese manufactured from this milk over the whole ripening (*10*). Comparison of the distribution of FFA in milk with the esterified FA highlighted some specificity in the release of FFA in milk. Short-chain and some medium-chain FFA (C4:0−C10:0) were released at a significantly higher percentage, compared to their proportion as esterified FA. The relative concentration of C4:0, in particular, was particularly high in milk (9.8% in milk FFA vs 4.0% in esterified FA). In contrast, the proportions of C14:0, C16:0, and C18:2 released in milk were significantly lower than their proportion in fat. The proportion of the three other FA, C12:0, C18:0, and C18:1, did not significantly differ in milk FFA and in total FA (**Figure 2**).

The distribution of FFA strongly differed in curd and in milk, with significantly lower proportions of short-chain FFA and higher proportions of long-chain FA in curd, compared to milk (**Figure 2**). The percentage of loss during curd drainage is illustrated in **Figure 3** for each individual FFA. More than 90% of the butyric (C4:0) and caproic (C6:0) acids of the milk and 80% of caprylic acid (C8:0) were lost in the whey during curd drainage. For the other FFA (from C10:0 to C18:2), losses ranged from 28 to 50%. The percentage loss did not depend on the presence of propionibacteria ($p > 0.01$).

Table 1. Concentrations in Individual Free Fatty Acids (FFA), in Milligrams per Gram of Fat, in Cheese Milk and during the Ripening of Swiss Cheeses Manufactured with or without *P. freudenreichii* Added as a Ripening Culture[a]

			ripening day					
FFA	milk	curd	10	17	24	31	46	day 46/curd ratio
			Without *P. freudenreichii*					
C4:0	0.136 a	0.012 f	0.012 f	0.027 e	0.041 d	0.055 c	0.085 b	7.0
C6:0	0.088 a	0.007 cd	0.002 d	0.007 cd	0.013 bc	0.014 bc	0.019 b	2.6
C8:0	0.089 a	0.018 b	0.018 b	0.018 b	0.018 b	0.019 b	0.021 b	1.2
C10:0	0.136 a	0.067 b	0.074 b	0.073 b	0.076 b	0.072 b	0.075 b	1.1
C12:0	0.158 a	0.090 c	0.101 bc	0.104 bc	0.106 bc	0.122 b	0.104 bc	1.2
C14:0	0.344 a	0.200 c	0.239 bc	0.248 b	0.260 b	0.254 b	0.251 b	1.3
C16:0	1.069 a	0.619 b	0.612 b	0.628 b	0.654 b	0.725 b	0.759 b	1.2
C18:0	0.603 a	0.273 b	0.278 b	0.286 b	0.315 b	0.323 b	0.284 b	1.0
C18:1	1.210 a	0.706 b	0.732 b	0.726 b	0.740 b	0.797 b	0.785 b	1.1
C18:2[b]	0.066 a	0.046 b	0.042 b	0.041 b	0.041 b	0.039 b	0.046 b	1.0
SCFFA[c]	0.313 a	0.037 e	0.033 d	0.052 de	0.071 cd	0.089 c	0.124 b	3.3
MCFFA[d]	0.639 a	0.357 c	0.415 bc	0.425 bc	0.441 b	0.448 b	0.429 bc	1.2
LCFFA[e]	2.949 a	1.643 b	1.664 b	1.681 b	1.750 b	1.885 b	1.874 b	1.1
total FFA	3.901 a	2.038 c	2.111 bc	2.158 bc	2.263 bc	2.422 b	2.427 b	1.2
			With *P. freudenreichii*					
C4:0	0.129 c	0.014 e	0.027 e	0.094 d	0.188 b	0.194 b	0.227 a	16.5
C6:0	0.080 c	0.005 e	0.011 e	0.036 d	0.091 b	0.094 b	0.109 a	24.1
C8:0	0.086 a	0.017 e	0.021 e	0.037 d	0.073 bc	0.064 b	0.085 ab	5.0
C10:0	0.131 c	0.066 e	0.071 e	0.101 d	0.208 b	0.229 b	0.257 a	3.9
C12:0	0.145 cd	0.088 d	0.098 d	0.173 c	0.313 b	0.369 ab	0.407 a	4.6
C14:0	0.301 de	0.201 e	0.222 de	0.345 d	1.046 c	1.319 b	1.517 a	7.5
C16:0	0.940 de	0.629 e	0.689 e	1.089 d	3.288 c	4.231 b	4.705 a	7.5
C18:0	0.515 d	0.268 f	0.295 ef	0.406 de	1.127 c	1.421 b	1.637 a	6.1
C18:1	1.037 e	0.717 f	0.785 f	1.257 d	3.024 c	3.273 b	3.778 a	5.3
C18:2	0.055 b	0.055 b	0.041 b	0.055 b	0.063 ab	0.103 b	0.167 a	3.9
SCFFA	0.295 c	0.035 e	0.059 e	0.168 d	0.352 b	0.352 b	0.421 a	11.9
MCFFA	0.577 de	0.355 f	0.391 ef	0.619 d	1.568 c	1.918 b	2.181 a	6.1
LCFFA	2.547 d	1.656 e	1.824 e	2.815 d	7.542 c	9.092 b	10.281 a	6.2
total FFA	3.419 d	2.046 e	2.273e	3.601d	9.462 c	11.361 b	12.883 a	6.3

[a] Different letters within the same row indicate significant differences ($p < 0.05$). [b] C18:2 = C18:2cis-9,12. [c] SCFFA (short-chain FFA), sum C4:0−C8:0. [d] MCFFA (medium-chain FFA), sum C10:0−C14:0. [e] LCFFA (long-chain FFA), sum C16:0−C18:2.

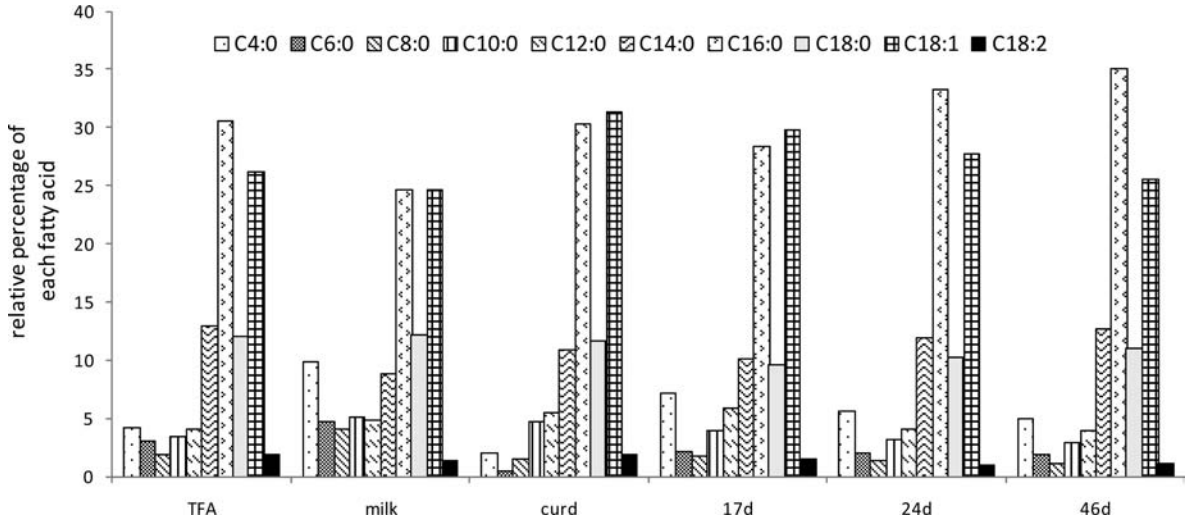

Figure 2. Distribution of individual fatty acids (FA, % mol/mol of total fatty acids) either esterified in cheese (total FA), or present as free fatty acids at various stages of manufacture and ripening of Swiss cheeses manufactured with added *P. freudenreichii*. Values are means of duplicate cheeses.

During ripening, the concentrations of most FFA increased, as shown in **Table 1**. The ratios of the concentration of FFA in ripened cheese and curd ranged from 1 to 24, depending on the presence of propionibacteria and the FA considered. In the control cheeses, the total amount of FFA increased by only 20% over the whole ripening period. Only C4:0 and, to a lesser extent, C6:0 significantly increased in concentration (7.0 and 2.6 times, respectively). In contrast, in prop-cheeses, the total FFA concentration increased by a factor of 6.3 over the ripening period, with a significant increase of all individual FFA (**Table 1**). Short-chain FFA showed the

highest increase (factor of 11.9), especially C4:0 and C6:0 (16.5 and 24.1, respectively). Medium- and long-chain FFA increased by a factor of ~6 over the same period. The global distribution of FFA was roughly comparable to the distribution of total fatty acids esterified in cheese fat, with palmitic (C16:0) and oleic (C18:1) acids representing 58 ± 4% (mol/mol) of total FFA, followed by myristic (C14:0) and stearic (C18:0) acids (11% each) (**Figure 2**). Some significant changes could, however, be observed in the distribution of FFA over the ripening period. During the first period of ripening, the relative concentration of C4:0 markedly increased, from 1.9% in curd to 7.2% at day 17. Later, during the period of rapid lipolysis, the relative proportions of C16:0 and C18:1 were inverted. They varied from 28.4 and 29.8%, respectively, at day 17, to 33.2 and 27.7% at day 24. Such an inversion restored the relative distribution of these two FA that was encountered in total FA esterified in cheese fat (**Figure 2**).

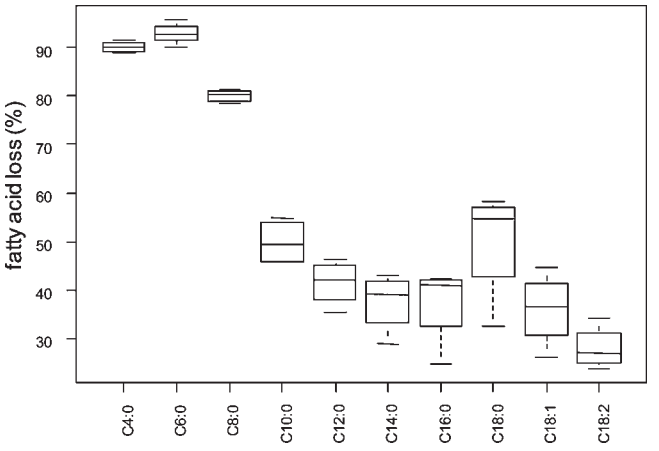

Figure 3. Loss of individual free fatty acids in whey during curd draining, expressed as a percentage of loss for each fatty acid: box plot of values of four cheeses.

To better visualize the specificity of release of each FFA, in particular the minor FFA, the percentage of each individual FA released (FAi) was also calculated, as previously reported (*10*), using the following formula: % individual FFA released = (FFAi/FAi) × 100. This percentage corresponds to the fraction of each individual FA that is released from glycerides during the ripening. The results are shown in **Figure 4**. The overall release of FFA was 1.51% mol of FFA/mol of FA over the entire ripening period (from curd to day 46 of ripening). During this period, the release of two FA, C4:0 and C16:0, was significantly greater in comparison to the other FA (2.11 and 1.78% of release, respectively). In contrast, the release of C18:2, C8:0, and C6:0 was less significant, with percentages of release of 0.57, 0.93, and 1.06%, respectively.

Significant differences were also observed between the release of FFA during the different periods of ripening considered. During the first period of ripening (from curd to day 17), the release of C4:0 was more significant (0.80%) than all the other FA. To a lesser extent, the release of C12:0 (0.37%) was significantly higher than that of C16:0, C14:0, C18, or C18:2 (< 0.20%). During the period of fast ripening (from day 17 to day 24), the releases of C16:0 and C4:0 (0.93 and 0.92% released, respectively) were more significant than those of C6:0, C8:0 C10:0, C12:0, and C18:2 (< 0.65%). C18:1, C14:0, and C18:0 showed intermediary levels of release (0.72−0.82%). During the end of ripening in the warm room (from day 24 to day 31), a greater release of C16:0 was detected (0.41%), whereas there was less release of the short-chain FFA (< 0.05%). During the period of ripening in the cold room (from day 31 to day 46), no significant differences were observed in the release of the FFA.

Lipid Classes. Lipid classes were investigated in the prop-cheeses, at three stages of ripening (indicated by the arrows in **Figure 1**, i.e., in the curd, at day 24, and at day 46), by TLC and HPLC. TLC gave a clear overview on the products of lipolysis (**Figure 5**), whereas HPLC provided an accurate quantification of the partial glycerides (**Figure 6**).

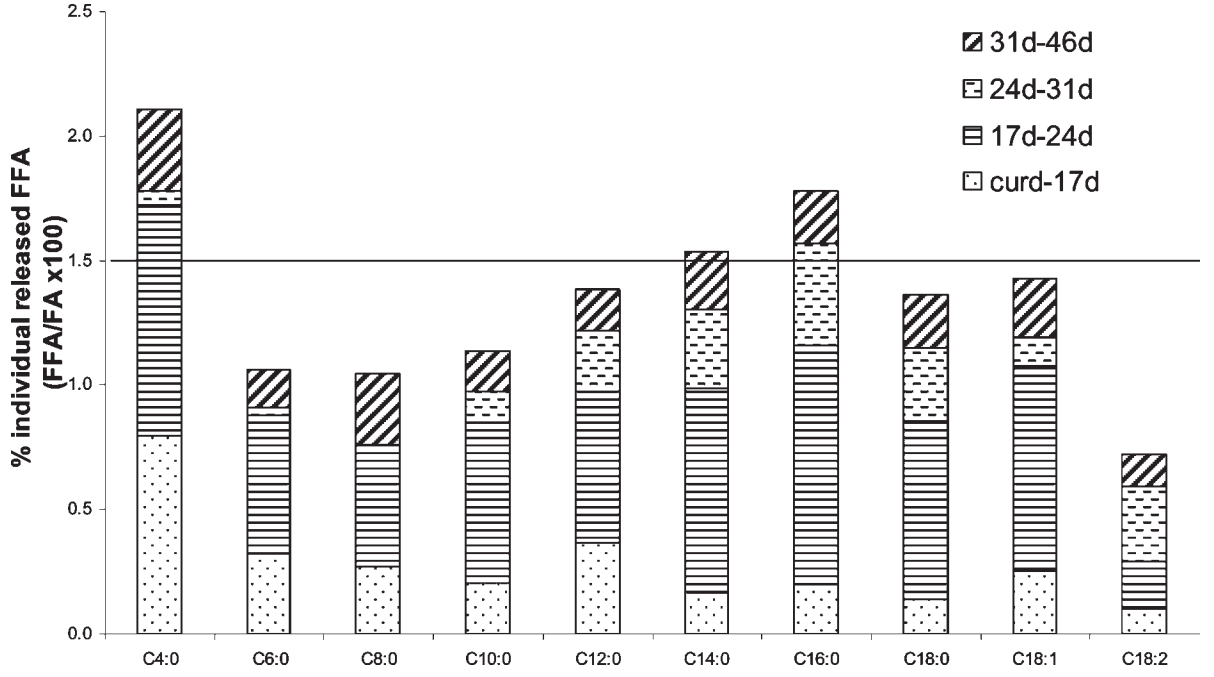

Figure 4. Percentage release of each fatty acid (FA) during ripening compared to the amount of this FA esterified in cheese. The line represents the percentage of global FA release, calculated as follows: sum of molar concentrations of FFA/sum of molar concentration of total fatty acids. Values are means of duplicate cheeses.

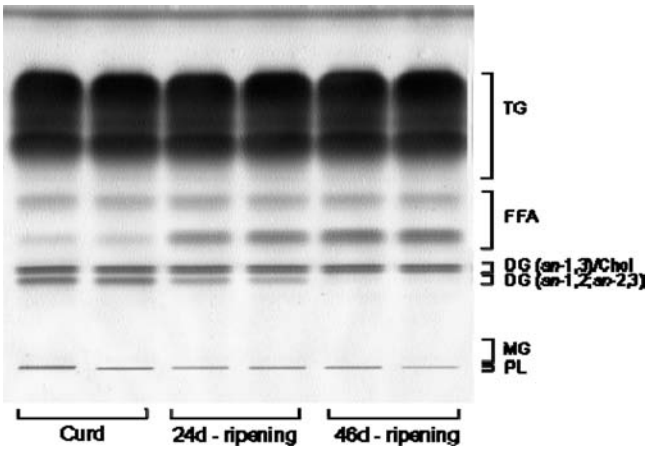

Figure 5. TLC displaying the evolution of lipid classes during lipolysis in Swiss-type cheese manufactured with *P. freudenreichii* as a ripening culture. Abbreviations: TG, triglyceride; FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; PL, polar lipids; Chol, cholesterol. For each stage of ripening two replicate samples were analyzed.

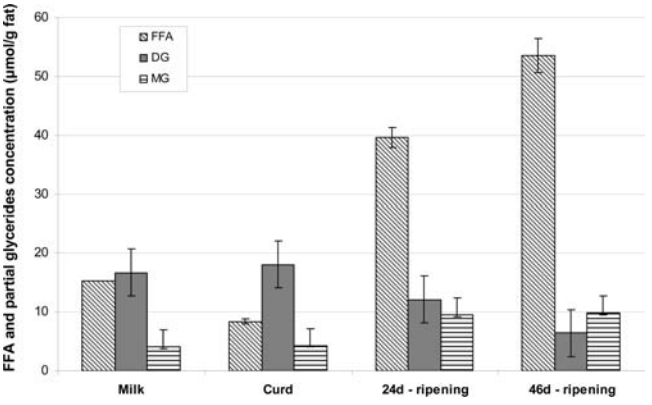

Figure 6. Quantification of the products of lipolysis (partial glycerides by HPLC and FFA by GC) during lipolysis in Swiss-type cheese manufactured with added *P. freudenreichii*. Error bars stand for standard deviation for FFA and for root mean square errors of prediction of the HPLC calibration curves for DG and MG.

The main class of lipids detected were TG, as expected (**Figure 5**). The TG had a heterogeneous acyl chain length, as shown by the two wide upper bands in the TLC banding pattern. DG (*sn*-1,3 and *sn*-1,2;2,3) and FFA resulting from early lipolysis were also clearly visualized. The two bands observed for FFA are explained by the difference in acyl chain length in milk fat. A band of low intensity was observed for short- to long-chain FFA in comparison to the very long chain FFA (20 carbon atoms or more). The low intensity of the upper band in the curd can be explained by a low level of initial lipolysis and a preferential loss of short-chain FFA in whey during curd draining. MG were not visualized by TLC, which indicates that they were not present or present only at low concentrations. During the ripening, the intensity of the lowest FFA band (C4−C18) increased, whereas the band corresponding to the DG (*sn*-1,2;2,3) decreased. These observations indicate that (i) different substrates (DG, MG) were hydrolyzed during ripening and (ii) the rates of hydrolysis of DG and MG were greater than that of TG, because the intensity of the DG band increased during ripening and MG species resulting from DG hydrolysis did not accumulate, even temporarily.

Partial glycerides were quantified by HPLC. The results are shown in **Figure 6**, along with the molar concentrations of the FFA quantified by GC. The concentrations of partial glycerides did not significantly differ in the milk and curd, in contrast to the concentrations of FFA. This result shows that partial glycerides remained in the curd during curd draining. DG and MG concentrations were 18 and 4 μmol/g of fat, respectively, in the curd. During ripening, the concentrations of FFA increased from 8 μmol/g fat in the curd to 54 μmol/g fat at the end of ripening (day 46). In comparison, the concentration of DG decreased from ~18 to 6 μmol/g of fat, whereas the concentration of MG showed a slight but nonsignificant increase. The FFA present in the system result from the hydrolysis of either (i) partial glycerides only or (ii) partial glycerides and TG. In the case of hypothesis i, the following equation can be applied from the principle of moles conservation over the ripening period:

$$\int_{ti}^{tf} \Delta\mathrm{FFA} \approx \int_{tf}^{ti} (\Delta\mathrm{DG} + 2 \times \Delta\mathrm{MG}) \qquad (1)$$

The results from **Figure 6** shows that eq 1 was not verified. On the contrary, in the Swiss cheese systems observed, the equation of moles conservation corresponded to

$$\Delta\mathrm{FFA} > \Delta\mathrm{DG} + 2 \times \Delta\mathrm{MG} \qquad (2a)$$

$$46 > 12\ \mu\mathrm{mol/g\ of\ fat} \qquad (2b)$$

This demonstrated that FFA were released from the hydrolysis of both partial glycerides and TG. In addition, the quasi constant MG concentration over the ripening period, compared to the decrease of DG concentration, indicated that DG were hydrolyzed more rapidly than MG.

DISCUSSION

The aim of the present study was to determine the time course and specificity of lipolysis and the respective involvement of the different lipolytic agents during Swiss cheese manufacture and ripening. For the first time, the same cheese samples were used to monitor both the growth of propionibacteria and the rate of lipolysis throughout the manufacture and ripening of Swiss cheese.

Respective Contributions to Global Lipolysis Occurring during Each Manufacture and Ripening Period. The final level of lipolysis in the cheeses inoculated with propionibacteria was 12.9 mg of FFA/g of fat and was similar to values previously reported for Swiss cheese: 13.4 mg/g of fat at 48−55 days of ripening (*10*), 14.1 mg/g of fat at 28−42 days of ripening (*3*), 11.4−14.7 mg/g of fat at 77 days of ripening (*15*), and 9−13 mg/g of fat (*2*).

The majority of lipolysis (71%) occurred during the ripening in the warm room and is in close agreement with a previous study reporting a value of 68% (*10*). The present study additionally shows that the maximum rate of lipolysis occurred after the period of maximal growth of propionibacteria, during the second week in the warm room, when 45% of the total FFA were released (from day 17 to day 24). From observation of **Figure 1**, it could be assumed that lipolysis by *P. freudenreichii* results from the activity of extracellular esterase(s), at least predominantly, rather than intracellular esterases released from lysed cells. This hypothesis is strengthened by the recent demonstration that *P. freudenreichii* possesses at least one secreted lipolytic esterase with activity on milk fat (*14*). The involvement of *P. freudenreichii* in cheese lipolysis clearly differs from that of cheese-related LAB, which possess only intracellular esterases, which are active on cheese fat only after their release from lysed cells (*1*, *23*, *24*). During the late ripening period at 4 °C (from day 31 to day 46), lipolysis continued, in agreement with a previous study (*10*).

From the data in **Table 1**, the rate of release of FFA was calculated to be 4 times lower at 4 °C than in the warm room at 24 °C. An increase in FFA was also observed in Swiss cheese during the first week of storage at 4 °C.

Early lipolysis occurring in milk is likely due to the activity of LPL, because the lactic starters begin to grow only in the curd and, as a result, their lipolytic enzymes cannot be responsible for the release of FFA in milk. The amount of FFA present in young curd (at day 1) represented 16% of the final amount of FFA, in agreement with a previous result in Swiss cheese (*10*). The activity of LPL can be affected by the quality of the milk and the heat treatment applied to milk, thus modulating the intensity of early lipolysis of milk (*7*).

In the control cheeses without propionibacteria, the final concentrations of FFA (2.4 mg/g of fat) were comparable to that of (semi)hard cheeses ripened with lactic acid bacteria only, as, for example, in Cheddar cheese, with 2.4−3.3 mg of FFA/g of fat at 56 days of ripening (*5*), and in Gouda cheese, with 5−8 mg of FFA/g of fat (*25*). The majority of lipolysis (84 ± 5%) occurred within the first day of manufacture (**Figure 1**). This is probably similar for many (semi)hard internally LAB ripened cheeses, for which the final level of lipolysis is low, and, consequently, the contribution of early lipolysis proportionally high. In Cheddar cheese, for example, it can be calculated from the data of Hickey et al. (*4*−*6*) that 73−90% of the amount of FFA of ripened (6-month-old) cheeses were already present in curd at day 1. This feature was observed regardless of the initial concentrations of FFA at day 1, which varied over large ranges (from 0.5 to 1.2 g of FFA/kg of cheese).

Specificity of Early Lipolysis in Cheese Milk. The FA composition of the milk and cheese used in this study was typical for milk fat, with C16:0 (32%) and C18:1 (27%) being the predominant FA and short-chain FA (C4:0−C8:0) ranging from 1.7 and 4.2% of total FA (*26*). In cheese milk, the release of C4:0 was proportionally greater than that of the other FFA. This early preferential release is likely to be due to the activity of the milk indigenous LPL (*7*), which exhibits an *sn*-1,3 regioselectivity and no typoselectivity. Because short-chain FA, and in particular C4:0, are predominantly at the *sn*-3 position of milk fat triglycerides, they are preferentially released by LPL. The second noticeable difference in the distribution of FFA in milk, compared to the distribution of total FA in fat, was the enrichment in oleyl (C18:1) moieties compared to palmityl (C16:0) ones. Such a distribution of FFA in milk has previously been reported (*27*, *28*) and could also result from the predominant *sn*-3 regiodistribution of oleic acid compared to palmitic acid, resulting in a higher release of oleic compared to palmitic acid by LPL.

Influence of Curd Draining on FFA and Partial Glyceride Concentrations. The extent and specificity of FFA loss during curd drainage were detailed for the first time in Swiss cheese in the present study. More than 90% of C4:0 and C6:0, 80% of C8:0, and 28−50% of the other FFA present in milk were lost during curd drainage. Similar results have been reported in Cheddar cheese, with losses of short-, medium-, and long-chain FFA during the Cheddar cheesemaking process of ∼77−86, 9−57, and 30−40%, respectively (*4*). The differences in the loss of FFA according to chain length are very likely due to their partitioning between the aqueous and lipid phases. For example, the log of the partition coefficients in octanol and water, log $P_{oct/wat}$, is equal to 0.79, 4.09, and 7.73 for butyric, lauric, and oleic acids, respectively.

In contrast, partial glycerides (MG, DG) generated during early lipolysis in milk were retained within the curd. Their initial concentrations were 1.4 ± 1.0 and 9.6 ± 2.3 mg/g of fat for MG and DG, respectively. These are in the range of concentrations previously reported for milk (MG, 0.8−3.8 mg/g of fat; DG, 2.8−22.5 mg/g of fat) (*26*, *29*). The presence of partial glycerides at the beginning of ripening is important, because they are substrates for the esterases of LAB, which are not able to hydrolyze TG (*12*). Therefore, as the initial levels of MG and DG in the curd are determined by early lipolysis, this is likely to influence the level and specificity of lipolysis that occur during ripening by the bacterial esterase activity. Accordingly, in Cheddar cheese, differences in FFA concentrations in cheese milk, likely related to differences in the concentrations of partial glycerides in the curd, were shown to highly influence the amounts of FFA released during the subsequent ripening stages (*5*).

The marked changes in the distribution of FFA in curd, compared to milk (**Figure 2**), have two consequences. On the one hand, the concentration of FFA in cheese milk, but not in curd, is an indicator of the amount of partial glycerides present at the beginning of the ripening. On the other hand, young curd, rather than cheese milk, should be considered as the reference point to calculate the release of FFA during ripening.

Respective Contributions of Lactic Acid Bacteria versus Propionibacteria to Global Lipolysis. This study has shed light on the predominant contribution played by *P. freudenreichii* lipolytic activity in Swiss cheese. If we hypothesize that the activity of LAB was similar in the presence and in the absence of added propionibacteria, it can be estimated that *P. freudenreichii* lipolytic enzyme(s) were responsible (i) for 96% of the amount of FFA released during ripening and (ii) for 81% of the total amounts of FFA produced, the remaining part being mainly due to the early lipolysis occurring in milk. This latter value is in agreement with a previous study carried out with three other *P. freudenreichii* strains, reporting that 77−81% of FFA in ripened experimental Swiss-type cheeses would be due to the activity of propionibacteria (*8*).

The lipolytic enzymes of both starter LAB and nonstarter LAB could be involved in the weak lipolysis observed in the control cheeses during the ripening, with increases of 0.28 and 0.50 mg of FFA/g of fat in the two control cheeses, that is, only 12 and 19% of the total amount of FFA released. The differences between the duplicate cheeses, manufactured from different batches of milk, may be due to differences in the composition of the nonstarter LAB microflora. Short-chain FFA, especially C4:0, were proportionally more significantly released than medium- and long-chain FFA in the control cheeses without propionibacteria, as observed in Cheddar cheese (*2*, *6*). It should be noted that C4:0 and C6:0 can also result from amino acid catabolism.

Specificity of Lipolysis Catalyzed by *P. freudenreichii* Lipolytic Esterase(s). The characterization of lipolysis products during ripening showed that the main lipolytic agent involved, *P. freudenreichii* lipolytic esterase(s), exhibited a low apparent specificity. First, all types of FA (from short to long, saturated and unsaturated) were released, and the evolution of the distribution of FFA at the different manufacture and ripening stages did not differ markedly from the distribution of FA esterified in fat (**Figure 2**). These features result from the activity of a nontyposelective esterase, without a strong regiospecificity. Second, the slight preferential release of C4:0 at the beginning of the ripening (36% of the total amount released before day 17 vs 12% for the other FFA, on average) and of C16:0 later during the warm room ripening period may indicate a slight *sn*-1,3 regiospecificity of the esterase(s) active during the ripening. In a previous paper on the lipolysis of Swiss cheese, four FA were found to be more significantly released in the warm room than the other FA: C4:0, C16:0, C18:1, and C18:3 (*10*). The preferential release of C4:0 is important because it has the lowest flavor threshold value among the FFA and thus highly contributes to

the global flavor of Emmental (Swiss cheese) (*1*). Finally, the results of this study show that the esterase is active on a broad range of substrates, with a slight apparent specificity for partial glycerides compared to TG and more specifically for DG compared to MG.

In summary, the apparent activity of *P. freudenreichii* lipolytic esterase(s) in Swiss cheese was characterized by no typoselectivity, a weak regiospecificity, and a broad range of glyceride substrates. These conclusions should, however, be considered cautiously, because the complex macromolecular organization of fat substrate in the cheese matrix may also influence the release in FFA. For instance, the fraction of crystallized fat, which is not accessible to the action of lipolytic esterases, is greater for long-chain unsaturated FA and may also contribute to modulate the apparent specificity of lipolytic enzymes in cheese. It may also induce some differences of apparent specificity depending on room temperature. This observation strengthens the requirement of testing the specificity of the lipolytic activity of *P. freudenreichii* lipolytic esterase on model substrates.

In conclusion, this study has brought new insights into the quantitative and qualitative contribution of the different lipolytic agents to lipolysis in Swiss cheese. Early lipolysis occurring in cheese milk is due mainly to LPL activity liberating short-chain FFA and represents 16% of the final amount of FFA in the ripened cheeses. The majority of medium- and long-chain FFA and all of the DG and MG released in milk were retained within the curd, whereas a large proportion of short-chain FFA were lost in the whey. During ripening, lipolysis was essentially due to the activity of *P. freudenreichii* and occurred with a low apparent specificity, even though there was an initial preferential release of butyric acid followed by palmitic acid. TG, DG, and MG were all hydrolyzed, partial glycerides being hydrolyzed more rapidly than TG. As a result, the degree of early lipolysis, by varying the amount of DG and MG generated, could influence the intensity and specificity of further lipolysis occurring during ripening.

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