JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Lipolysis during Ripening of Emmental Cheese Considering Organization of Fat and Preferential Localization of Bacteria

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This study followed the progression of lipolysis in Emmental cheese by quantifying the concentrations of individual free fatty acids (FFA) released during ripening in each of the different rooms: 12 days at 12 °C, 28 days at 21 °C, and 8 days at 4 °C. Lipolysis, which corresponded to 1.56% of fat, mainly occurred in the 21 and 4 °C rooms, with 68 and 16.5% of total FFA, respectively. The nonselectivity of lipolytic enzymes was evidenced: all fatty acids were released with level of ≥1%. Differential scanning calorimetry experiments showed that the thermal properties of cheese were affected by (i) lipolysis of fat, that is, the monoacylglycerols, diacylglycerols, and FFA that may be localized at the fat/whey interface, and/or by (ii) hydrolysis of high-melting-point triacylglycerols constituted mainly by long-chain saturated fatty acids (e.g., palmitic acid). Analysis of the cheese microstructure was performed using confocal laser scanning microscopy. Fat globules were mainly disrupted after pressing of curd grains, leading to the release of the milk fat globule membrane (MFGM); fat inclusions were surrounded by pockets of whey, delimited by casein strands. Moreover, colonies of bacteria were preferentially localized in situ at the fat/protein interface. This study showed that both the localization of bacteria and the supramolecular organization of fat which was not protected by the MFGM can help the accessibility of milk fat to lipolytic enzymes and then contribute to the quality of cheese.

KEYWORDS: Lipolysis; cheese; milk fat; bacteria; crystallization

INTRODUCTION

Emmental cheese is probably the best-known Swiss-type cheese and is the most consumed hard cheese in France (253 000 t produced in 2004). Thus, it is of economic interest to improve both its functional and its sensorial properties by controlling ripening.

Cheese ripening involves a concerted series of microbiological, chemical, and biochemical changes that are ultimately responsible for the development of the characteristic flavor, texture, and appearance of individual cheese varieties (*1*). There are three primary routes by which biochemical activity proceeds in cheese during ripening, namely, proteolysis, lipolysis, and the metabolism of residual lactose and of lactate and citrate (*2*). The focus of research has primarily been restricted to the study of proteolysis, despite the significant role played by lipolysis in the biogenesis of cheese flavor. Furthermore, in the case of Emmental cheese, where the level of lipolysis is moderate during ripening, the contribution of lipolytic end products to cheese quality and flavor has received relatively little attention.

Milk fat is mainly composed of triacylglycerols that are esters of glycerol and fatty acids, which represent up to 98% of total lipids. The range of fatty acid chain lengths and degree of unsaturation are responsible for the complex composition of milk fat; >400 fatty acids and 200 triacylglycerols have been previously identified (*3*).

Lipolysis in cheese is due to the presence of lipolytic enzymes called hydrolases (lipases and esterases) that cleave the ester linkage between a fatty acid and the glycerol moiety of the triacylglycerol. Hydrolysis of triacylglycerols produces free fatty acids (FFA) with chain lengths ≥C4, glycerol, and mono- and diacylglycerols. Also, short-chain fatty acids can be produced from the metabolism of carbohydrates and amino acids by the bacterial flora (*4*). FFA are important precursors of catabolic reactions, which produce a variety of volatile compounds such as alcohols, esters, aldehydes, ketones, and lactones, which contribute to cheese flavor (*4*, *5*). The levels of FFA and the development of cheese flavor stem directly from both the enzymatic and biochemical activities during ripening.

As for Cheddar cheese (*6*), the extent of lipolysis in Emmental cheese is normally low, that is, 1−2% of fat (*7*, *8*). However, it is essential for a good balanced flavor. Lipolysis does not need to be very extensive to make a distinct contribution to the sensory properties of Emmental cheese. Short- and intermediate-chain, even-numbered fatty acids (C4:0−C12:0) have low flavor thresholds, and each has a characteristic flavor note. FFA can also act as flavor precursors for many flavor compounds (*9*).

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10.1021/jf060214l CCC: $33.50
Published on Web 07/15/2006

The flavor and texture of cheese may be modified by the composition and amount as well as the supramolecular organization of the cheese fat. It is not yet clear whether the lack of flavor in low-fat cheeses is due to the lack of flavor precursors, which derive from the fat, the lack of fat as a solvent for flavor compounds, or the different physical structures of the reduced-fat cheese, which may reduce the rate of some enzymatic reactions essential to the formation of flavor compounds (*10*).

Fat is present in bovine milk in the form of small droplets, called milk fat globules, the size distribution of which ranges from 0.2 to 15 μm with an average diameter of 4 μm (*11*). Natural milk fat globules are surrounded by a biological membrane, the milk fat globule membrane (MFGM), the composition of which depends on the secretion process (*12*). The MFGM is organized as a trilayer and represents a physical barrier that protects the fat from lipolytic action. Previous studies have considered the effect of fat globule size on the levels of FFA at the end of cheese ripening, using homogenization (*13*), gravity separation (*14*), and microfiltration (*15*, *16*). Selection of fat globules of different sizes by gravity separation and microfiltration preserves the MFGM. Decreasing the size of fat globules by homogenization increases the milk fat surface area available for lipase activity and changes the composition of the surface due to the disruption of the MFGM and adsorption of proteins onto the newly formed surface, for example, caseins and whey proteins.

Information on the supramolecular structure of milk fat in dairy products is scarce. However, the organization of fat may be altered during the processing of milk and the manufacture of dairy products due to the mechanical and thermal treatments applied (*11*). Using confocal laser scanning microscopy, Lopez et al. (*17*) showed the disruption of fat globules during pressing of Emmental cheese grain curds. The authors showed that, at the end of ripening, fat is organized as (i) coalesced fat globules, (ii) aggregates of fat globules, and (iii) nonglobular fat, also called free fat (i.e., fat not protected by a MFGM). Moreover, Wijesundera et al. (*18*) reported that the shape of fat globules and the composition of the fat/protein interface may influence lipolysis and flavor-generating reactions which occur during ripening.

In Emmental cheese, the main source of lipases is the microbial flora. In particular, propionic acid bacteria are well-known for their higher lipolytic activity compared to lactic acid bacteria (*8*). Laloy et al. (*19*) reported that the microstructural and physicochemical dynamics of fat globules in cheese appear to influence the localization and retention of bacteria in cheese. Using electron microscopy, the authors localized the bacteria on the periphery of fat globules (*19*−*21*). However, to date, no detailed study has been undertaken to elucidate the mechanism of the accessibility of fat in cheese to lipolysis (*9*).

The objective of this study was to quantify the total and individual FFA levels after each of the main stages involved in the ripening of Emmental cheese. Furthermore, this work intends to increase the knowledge of the mechanisms involved in lipolysis, in particular the accessibility of lipolytic enzymes to fat, by characterizing in situ the organization of fat and the localization of bacteria in the cheese matrix.

MATERIALS AND METHODS

Emmental Cheese Manufacture. Three separate trials of Emmental cheese were manufactured in March, from separate milk batches, using a specialized pilot plant based in the INRA research center, Rennes (Chalon Megard, La Cluse, France).

Day P−*1*. Raw whole milk, purchased from a local dairy plant (Triballat, Noyal-sur-Vilaine, France), was collected the day before Emmental cheese production (P−1). The milk was standardized to a fat content/total nitrogen ratio (fat/TN) of 0.86 using skimmed milk and then stored overnight at 4 °C.

Day P. Standardized milk was thermized using a pilot pasteurizer Actijoule at 63 °C for 20 s (Actini, Evian les Bains, France) on the day of manufacture of the cheese (P). The cheese milk (850 L) was heated to 31 °C in a temperature-controlled milk vat. A lysozyme solution (Delvosyme, Gist Brocades, Seclin, France) was added to milk at a concentration of 0.01 mL kg^{-1}. The milk was supplemented with 0.01 mL kg^{-1} of a 510 g L^{-1} $CaCl_2$ solution. Milk was inoculated as follows: lactococci (EZAL MM100, Rhodia, Dangé-Saint-Romain, France) *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, and *Lc. lactis* subsp. *lactis* biovar *diacetylactis* [2×10^4 colony-forming units (CFU) mL^{-1})]; thermophilic lactobacilli (LH 100, Rhodia) *Lactobacillus delbrueckii* subsp. *lactis* and *Lb. helveticus*, both grown at 42 °C for 6 h on Phagex medium (Standa) and then inoculated into the milk at 1×10^5 CFU mL^{-1}; streptococci (PAL ITG ST 82-87, Standa) *Streptococcus thermophilus*, first grown at 42 °C for 4 h on Marstar 412A medium (Rhodia) and then inoculated into the milk at 1×10^6 CFU mL^{-1}; and propionic acid bacteria (PAL ITG P9, Standa) *Propionibacterium freudenreichii* subsp. *shermanii* (2×10^5 CFU mL^{-1}). After 30 min of incubation at 31 °C, milk pH was adjusted to 6.62 using CO_2 dissolved directly in milk. Liquid calf rennet diluted by a factor 2 in distilled water to ensure more homogeneous dispersion was added at 0.25 mL kg^{-1} (IMCU: 145, Berthelot, ABIA S.A., Meursault, France). A curd gel formed after 22 min and was hardened for 4−5 min (20% of the clotting time) and cut. The curd grains were mixed and heated for 20 min to 51 °C. Curd was drained off under vacuum (30 kPa) in the racking unit and molded at 47 °C in a 780-mm-diameter and 27-mm-high mold (Doryl). The curd was pressed for 4 h at 0.4 kPa (T_{room} = 24 °C). After pressing, the curd was turned over and acidified for 19 h in a temperature-controlled room at 24 °C.

Day P+1. The curd was removed from the molds, weighed, and placed in a cold brine bath for 48 h (saturated NaCl solution: 350 g L^{-1}; 12 °C; pH 5.2).

P+3 < Day ≤ P+52. Cheese was ripened for 12 days at 12 °C and 85% relative humidity, followed by 28 days at 21 °C and 80% relative humidity, and finally for 8−15 days at 4 °C, for a total ripening time of 48−55 days. Three Emmental cheeses of ≈80 kg each were manufactured in total.

Physicochemical and Microbiological Analysis. Cheese samples were analyzed after 1 and 52 days (P+1, P+52) for compositional analysis. At P+1, cylindrical samples (15 cm, ≈80 g) were taken from the cheese surface and at half-ray of the cheese, using a specific tool (ITFF, Rennes, France). At P+52, a sample of 20 × 6 × 4 cm (≈500 g) was cut at half-ray. Dry matter (DM) was measured by drying 2 g of cheese mixed with sand at 102 °C (±2 °C) for 7 h (*22*). Fat content was determined using the SBR method (*23*). Total nitrogen (TN) in cheese was determined using the Kjeldahl method (*24*). Proteolysis was measured by the evolution of soluble nitrogen at pH 4.6 (NCN, non-casein nitrogen) and 12% TCA-soluble nitrogen (NPN, non-protein nitrogen), according to the method previously described (*25*). To quantify the evolution of proteolysis, the following ratios were calculated: NCN/TN and (NCN − NPN)/TN, expressed as percentage of cheese TN content. Fat in dry matter (FDM, percent) was calculated as follows: FDM = fat/DM × 100. All analyses were performed in triplicate. The pH was measured at days 1 and 52.

For the determination of total fatty acids at each stage of ripening, a sample of Emmental cheese (15 cm, ≈80 g) was taken from the cheese surface, at half-ray and half-height, using a specific tool (ITFF). Samples of cheeses were stored at −80 °C until use and thawed at 4 °C for 24 h before analysis. Fat was extracted from cheese samples, and total fatty acids were methylated and analyzed as described in Lopez et al. (*26*).

Propionibacteria were enumerated on Pal Propiobac (Standa, Caen, France) (*27*), incubated anaerobically for 6 days at 30 °C. Nonstarter lactic acid bacteria (NSLAB) were enumerated on FH agar (*28*), incubated anaerobically for 72 h at 37 °C. Thermophilic streptococci were enumerated on M17 agar (*29*) containing lactose (20 g L^{-1}) (Biokar, Beauvais, France), incubated aerobically for 48 h at 42 °C.

Thermophilic lactobacilli were enumerated on MRS agar (Difco, Detroit, MI) acidified to pH 5.4 with acetic acid (*30*), incubated anaerobically for 48 h at 42 °C.

FFA Analysis. After each main stage of ripening, samples of Emmental cheese (15 cm, ≈80 g) were taken from the cheese surface, at half-ray and half-height, using a specific tool (ITFF). Samples of cheeses were stored at −80 °C until required for analysis and then stored at 4 °C during 24 h before analysis. Extraction of cheese lipids, isolation of FFA, and determination of FFA concentration by gas chromatography were performed as described by De Jong and Badings (*31*). Two independent extractions of cheese lipids were performed, and each sample was analyzed in duplicate per extraction. Isolation of FFA was performed using aminopropyl solid-phase extraction columns (500 mg 3 mL^{-1}; Phenomenex, Torrance, CA) and a vacuum manifold Maclerey Nagel (Duren, Germany). A Varian CP-3800 gas chromatograph equipped with an automatic on-column injector CP-8200 and a flame ionization detector (FID) was used with a fused silica capillary column BP21 (SGE) (length = 25 m; diameter = 0.53 mm; thickness = 0.5 μm). Direct cold on-column injection took place at 65 °C at a rate of 200 °C min^{-1}, then held at 250 °C for 1 min followed by cooling to 65 °C at a rate of 200 °C min^{-1}, and held for 20 min at 65 °C. The oven temperature was programmed from 65 to 240 °C at a rate of 10 °C min^{-1}. The FID temperature was 250 °C. The flow rate of the carrier gas (hydrogen) was 9.7 mL min^{-1} at 65 °C.

Identification of the individual fatty acids of the cheeses was based on a comparison of the retention times of the unknown FFA with those obtained from known FFA standards (Sigma, Steinheim, Germany) under identical conditions.

Quantification of the FFA levels of cheese samples was performed using a standard curve determined by injection of 0.5 μL of each external standard (500−5 ppm) under identical conditions as the samples and processing the chromatograms with the star GC workstation version 5.3 (Varian, Les Ulis, France). Three internal standards (C5:0, C11:0, and C17:0, 0.5 mg mL^{-1}) were added to each sample before centrifugation (1 mL per sample) to correct for possible losses of FFA during extraction.

Thermal Properties. The thermal properties of Emmental cheese were monitored by differential scanning calorimetry (DSC) using a TA Q-1000 calorimeter (TA-Instruments, Saint-Quentin-en-Yvelines, France). Calibration was performed using indium standard: melting point = 156.66 °C; ΔH melting = 28.41 J g^{-1}. About 50−70 mg of cheese was accurately weighed in a 100 μL hermetically sealed stainless steel pan. An empty hermetically sealed stainless steel pan was used as reference. Samples were (i) heated to 60 °C to melt all existing nuclei, (ii) cooled from 60 to −5 °C at −2 °C min^{-1}, and (iii) heated from −5 to 60 °C at 2 °C min^{-1}. Measurements were performed in triplicate on independent cheese samples.

Microstructure Analysis and Preferential Localization of Bacteria. Emmental cheese microstructure and the localization of bacterial colonies were determined using confocal laser scanning microscopy (CLSM) after each of the main stages involved during ripening. Thin slices of cheese, measuring approximately 5 mm × 5 mm × 3 mm, were prepared from freshly cut samples using a scalpel. The protein network and DNA of bacteria were stained using Acridine Orange fluorescent dye (Aldrich Chemical Co., Inc., Milwaukee, WI). A lipid-soluble Nile Red fluorescent dye (Sigma-Aldrich, St. Louis, MO) was used to label fat. Each slice of cheese was placed between a microscope slide and a cover slip. Cheese slices were incubated with the stains for 30 min in the dark at 4 °C. Microstructural analysis was performed using a confocal Leica TCS NT microscope (Leica Microsystems, Heidelberg, Germany), which employed an argon/krypton laser in dual-beam fluorescent mode, with excitation wavelengths of 568 nm for fat and 488 nm for both proteins and DNA. The depth of optical sectioning, which depends on the optical density of the samples, varied from about 100 to 250 μm from the surface of the sample. The two-dimensional images had a resolution of 1024 × 1024 pixels, and the pixel scale values were converted into micrometers using a scaling factor. For each of the main stages involved during the ripening, at least five independent samples of cheese were subjected to CLSM. For each cheese sample, at least five images were taken. Each micrograph presented was considered by the authors to be representative of the different cheese samples analyzed.

Table 1. Composition of Emmental Cheeses after 1 and 52 Days (End of Ripening)[a]

components	1 day	52 days
fat (g kg^{-1})	291.83 ± 5.48	298.33 ± 2.36
fat in dry matter (% w/w)	47.73 ± 0.85	47.56 ± 0.81
TN (g kg^{-1})	275.47 ± 1.86	276.03 ± 1.04
NCN (g kg^{-1})	12.07 ± 1.40	46.87 ± 0.90
NPN (g kg^{-1})	3.93 ± 0.93	38.6 ± 1.22
DM (g kg^{-1})	611.27 ± 0.75	627.67 ± 5.70
NCN/TN (% w/w)	4.38 ± 0.52	16.98 ± 0.38
(NCN − NPN)/TN (% w/w)	2.95 ± 0.83	3.00 ± 0.25
pH	5.16 ± 0.03	5.64 ± 0.03

[a] Results shown are the average of three independent trials (mean values ± standard deviation) Abbreviations: TN, total nitrogen; NCN, non-casein nitrogen; NPN, non-protein nitrogen; DM, dry matter.

Statistical Analysis. Analyses of variance (ANOVA) were performed using the General Linear Model procedure of Statgraphics Plus version 5 (Statistical Graphics Corp., Englewood Cliffs, NJ) to determine (i) the effect of the stage of ripening of Emmental cheese on the concentration of individual FFA and (ii) the effect of the concentration of FFA on the initial temperature of crystallization and the final temperature of melting. Differences between the treatment means were compared at the 5% level of significance using Fisher's least significance difference (LSD) test.

RESULTS AND DISCUSSION

Compositional Analysis and Levels of Bacterial Populations. The composition of Emmental cheeses at 1 and 52 days is shown in **Table 1**. At 52 days (end of ripening), the composition of the cheeses is within specified limits for Emmental cheese (*32*). As expected, the age-related increase in peptide and amino acid content (NCN, NPN) indicating the progression of proteolysis during ripening resulted in a decrease in the content of intact casein, that is, pH 4.6 insoluble nitrogen (**Table 1**). The fat in dry matter ratio and total nitrogen did not increase during ripening. However, the composition of fat changed due to lipolysis (see below). The decrease in the pH of the curd from 6.62 on rennet addition to 5.16 at 1 day was related to the production of lactic acid from lactose fermentation. The pH at 52 days of ripening increased to 5.64. In Emmental cheese the pH variation is related to the fermentative activity of propionibacteria, which convert lactate to propionate, acetate, and CO_2. Moreover, increases in pH during ripening of some hard cheeses are thought to be associated with decarboxylation and deamination of amino acids (*33*).

Bacterial populations were enumerated during the ripening of Emmental cheese (**Table 2**). At 52 days, we found 2.6 × 10^7 CFU g^{-1} of thermophilic streptococci, 1.3 × 10^8 CFU g^{-1} of thermophilic lactobacilli, 10^8 CFU g^{-1} of NSLAB, and 2.5 × 10^9 CFU g^{-1} of propionic acid bacteria. The results are consistent with previous reports in the literature (*7*, *34*). The kinetics of microbial growth occurring during ripening of Emmental cheese have been studied in detail by Thierry et al. (*34*), by enumerating both starter (thermophilic lactic acid bacteria and propionic acid bacteria) and nonstarter (lactic acid bacteria) flora. Steffen et al. (*7*) found that populations of lactic acid bacteria starters (thermophilic lactobacilli and streptococci) increase during the pressing step and decrease during the ripening from (2−3) × 10^8 to 10^8−10^5 CFU g^{-1}. Populations of NSLAB increase from the beginning of ripening and reach levels as high as 10^8 CFU g^{-1} (*7*). Propionic acid bacteria

Table 2. Levels of Bacterial Populations (Colony-Forming Units per Gram of Cheese) during Ripening of Emmental Cheeses

stage of cheese ripening	thermophilic streptococci	thermophilic lactobacilli	NSLAB[a]	propionic acid bacteria
end of 12 °C room period	$3.0 \times 10^8 \pm 4.2 \times 10^7$	$2.0 \times 10^8 \pm 3.1 \times 10^7$	$5.0 \times 10^5 \pm 2.1 \times 10^4$	$2.4 \times 10^7 \pm 1.2 \times 10^6$
after 19 days in the 21 °C room	$2.4 \times 10^8 \pm 2.1 \times 10^6$	$2.0 \times 10^8 \pm 1.1 \times 10^7$	$2.0 \times 10^8 \pm 1.3 \times 10^7$	$1.6 \times 10^9 \pm 1.4 \times 10^8$
end of ripening (52 days)	$2.6 \times 10^7 \pm 2.1 \times 10^6$	$1.3 \times 10^8 \pm 4.1 \times 10^7$	$1.0 \times 10^8 \pm 1.3 \times 10^7$	$2.5 \times 10^9 \pm 1.2 \times 10^8$

[a] NSLAB, non-starter lactic acid bacteria.

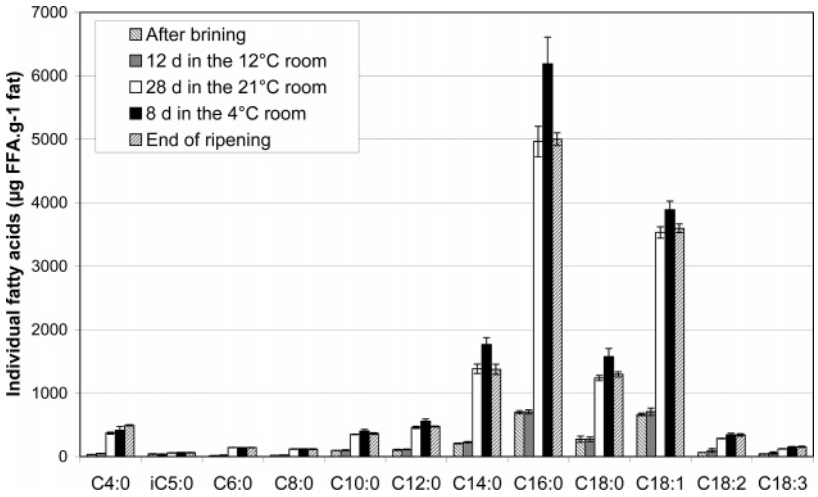

Figure 1. Concentrations of individual free fatty acids, expressed in micrograms of FFA per gram of fat, after the main stages involved during the ripening of Emmental cheese. ANOVA analysis is presented in **Table 3**.

develop in the warm room (e.g., 21−24 °C) to typically achieve levels of $\approx 10^9$ CFU g^{-1} by the end of ripening (*7*).

Assessment of Lipolysis during Ripening. The total FFA content measured in the curd at 1 day was 646 ± 133 μg of FFA g^{-1} of cheese and did not increase significantly (LSD test, $\alpha < 0.05$) after brining: 656 ± 12 μg of FFA g^{-1} of cheese. The variable level of lipolysis that may be detected after manufacture depends mainly on the characteristics of the milk used: stage of lactation, type of milking collection, and cooling and agitation in farm tanks, which may act on the activity of the lipoprotein lipase and thus on milk fat enzymatic hydrolysis (*35*). The thermization of milk (63 °C, 20 s) as well as the cooking of Emmental curd grains (51 °C for 20 min) inactivates the lipoprotein lipase as established by Driessen (*36*). After storage in the 12 °C room, the total content of FFA, 708 ± 57 μg g^{-1} of cheese, did not increase significantly (LSD test, $\alpha <$ 0.05). FFA content increased significantly to 3844 ± 148 μg g^{-1} of cheese after 28 days at 21 °C and to 4605 ± 283 μg of FFA g^{-1} of cheese after 8 days in the 4 °C room. The significant increase of FFA observed in the 21 °C room was concomitant with the growth of NSLAB and propionic acid bacteria (**Table 2**). Storage in the 4 °C room for an additional 7 days decreased significantly the FFA content to 3958 ± 49 μg g^{-1} of cheese (LSD test, $\alpha < 0.05$). This decrease in total FFA content at the end of ripening was considered to be due to the transformation of FFA to flavor compounds. The level of lipolysis, measured as maximum release of FFA after 8 days in the 4 °C room, is 4.6 g kg^{-1} of cheese, reflecting a low level of lipolysis, and is in agreement with levels of lipolysis previously reported in Emmental cheese, for example, on the order of 2−7 g kg^{-1} of cheese (*7*, *8*). During ripening the amount of total FFA released is increased by a factor of ≈7. The total FFA measured after 8 days in the 4 °C room corresponded to 15.55 mg of FFA g^{-1} of fat in Emmental cheese (1.56% fat). These results are similar to those of Berdagué et al. (*37*), who reported 4.2 g of FFA kg^{-1} of cheese, which corresponded to 14 mg of FFA g^{-1} of fat (1.4% fat) in Emmental "Grand Cru". Excessive lipolysis is considered to be undesirable, and moderate levels of FFA may be considered as rancid by some consumers (*1*).

The concentrations of each of the 11 individual FFA, from butyric (C4:0) to linolenic (C18:3) acids, are shown in **Figure 1** and **Table 3**. Irrespective of ripening stage, palmitic acid (C16:0), oleic acid (C18:1), myristic acid (C14:0), and stearic acid (C18:0) were present at the highest concentrations and constituted 80−86% of the total FFA, as in milk fat (*3*). The same most abundant FFA released during ripening of Emmental cheese were found in previous studies (*8*, *37*, *38*). The concentration of total FFA increased significantly ($P < 0.001$) during storage of Emmental cheese for 28 days in the 21 °C room and for 8 days in the 4 °C room (**Table 3**). The concentration of short-chain FFA [Σ(C4:0−C8:0)] increased significantly ($P < 0.001$) after each ripening stage, from 68 ± 4 to 753 ± 18 μg g^{-1} of fat (**Table 3**). The concentration of medium- [Σ(C10:0−C14:0)] and long-chain [Σ(C16:0-C18:3)] FFA increased significantly ($P < 0.001$) during storage for 28 days at 21 °C and then storage for 8 days in the 4 °C room (**Table 3**).

The concentration of the individual FFA that were specifically released during each ripening stage, for example, in the 12 °C room, in the 21 °C room, and in the 4 °C room, were calculated and are presented in **Table 4**. If the total FFA content after 8 days in the 4 °C room is considered to be the maximum FFA released during ripening, this suggests that 14.3 ± 0.8% of FFA were released from cheese milk after brining. Only 0.9 ± 0.1% of total FFA content was released during storage at 12 °C for 12 days, which corresponded to 12.4 μg of FFA g^{-1} of fat per day. The greatest content of FFA was released in the 21 °C room, that is, 68.2 ± 7.6% of total FFA. Finally, 16.6 ± 1.7% of total FFA were released in the 4 °C room (**Table 4**). The expression of these results as a function of time showed that, per day, 378.6 μg of FFA g^{-1} of fat was released in the 21 °C room, whereas 321.9 μg of FFA g^{-1} of fat was released in the 4 °C room, which corresponded to a decrease of only ≈15% in the lipolytic activity at 4 °C compared to that at 21 °C.

Table 3. Concentrations of Individual Free Fatty Acids from Butyric Acid (C4:0) to Linolenic Acid (C18:3) during Ripening of Emmental Cheese[a]

	individual FFA concentration during ripening (μg g^{-1} of fat)					
fatty acid	after brining	end of 12 °C room period	end of 21 °C room period	8 days in the 4 °C room	end of ripening	stat[b]
C4:0	32a ± 1	50a ± 1	370b ± 14	415b ± 58	493c ± 10	***
C6:0	14a ± 3	21b ± 6	143c ± 2	142c ± 1	142c ± 3	***
C8:0	22a ± 0	25a ± 2	119b ± 5	123b ± 2	118b ± 7	***
C10:0	97a ± 3	100a ± 8	350b ± 5	402c ± 27	363b ± 15	***
C12:0	106a ± 8	112a ± 6	461b ± 18	557c ± 37	474b ± 7	***
C14:0	207a ± 7	227a ± 11	1384b ± 74	1766c ± 109	1375b ± 81	***
C16:0	699a ± 25	705a ± 32	4965b ± 240	6187c ± 424	5001b ± 100	***
C18:0	274a ± 47	272a ± 36	1238b ± 41	1574c ± 128	1296b ± 41	***
C18:1	664a ± 21	708a ± 59	3532b ± 89	3888c ± 138	3597b ± 69	***
C18:2	66a ± 3	96a ± 29	287b ± 6	344c ± 25	342c ± 20	***
C18:3	41a ± 3	55a ± 19	122b ± 4	148c ± 12	155c ± 12	***
Σ(C4:0–C8:0)	68a ± 4	96b ± 5	632c ± 22	680d ± 60	753e ± 18	***
Σ(C10:0–C14:0)	410a ± 18	439a ± 25	2195b ± 96	2725c ± 172	2212b ± 71	***
Σ(C16:0–C18:3)	1744a ± 99	1836a ± 173	10144b ± 375	12141c ± 725	10391b ± 173	***
Σ(C4:0–C18:3)	2222a ± 126	2371a ± 193	12971b ± 491	15546c ± 957	13356b ± 167	***

[a] The sums of the concentrations of short- [Σ(C4:0-C8:0)], medium- [Σ(C10:0-C14:0)], and long-chain [Σ(C16:0-C18:3)] FFA were calculated. Results shown are the average of three independent trials (mean values ± standard deviation). Values in the same row with the same letter are not significantly different according to the LSD test ($\alpha < 0.05$). [b] Probability of *F* test: ***, $P < 0.001$.

Table 4. Concentrations of Individual Free Fatty Acids from Butyric Acid (C4:0) to Linolenic Acid (C18:3) Released during Each Stage of Ripening[a]

	FFA (μg g^{-1} of fat)					% FFA				
fatty acid	after brining	12 days in the 12 °C room	28 days in the 21 °C room	8 days in the 4 °C room	stat[b]	after brining	12 days in the 12 °C room	28 days in the 21 °C room	8 days in the 4 °C room	stat[b]
C4:0	32ab ± 0	18a ± 0.4	320c ± 18	45b ± 7.6	***	1.4a ± 0.03	12.2b ± 1.5	3.0c ± 0.2	1.8ac ± 0.1	***
C6:0	14a ± 3	7a ± 3	123b ± 34	–2a ± 0.2	***	0.6a ± 0.05	4.4b ± 1.5	1.2a ± 0.2	–0.1a ± 0.0	***
C8:0	22a ± 0	3b ± 0.2	95c ± 11	4b ± 0.23	***	1.0a ± 0.03	1.9b ± 0.2	0.9a ± 0.0	0.1c ± 0.0	***
C10:0	97a ± 3	3b ± 0.2	250c ± 23	52d ± 4.2	***	4.4a ± 0.06	1.9b ± 0.2	2.4b ± 0.0	2.0c ± 0.1	***
C12:0	106a ± 8	5b ± 0.6	349c ± 31	96a ± 9.6	***	4.8a ± 0.05	3.7b ± 0.1	3.3c ± 0.1	3.7b ± 0.0	***
C14:0	207a ± 7	20b ± 1.6	1157c ± 115	382d ± 43	***	9.3a ± 0.11	13.6b ± 0.9	10.9c ± 0.1	14.8b ± 0.1	***
C16:0	699a ± 25	6b ± 0.4	4261c ± 396	1221d ± 141	***	31.5a ± 0.35	3.8b ± 0.3	40.2c ± 0.8	47.4d ± 0.5	***
C18:0	274a ± 47	–3b ± 0.8	966c ± 159	336a ± 38	***	12.3a ± 0.78	–1.7b ± 0.3	9.1c ± 0.5	13.1a ± 0.1	***
C18:1	664a ± 21	44b ± 4	2825c ± 305	356d ± 21	***	29.9a ± 0.40	29.4a ± 1.5	26.6b ± 0.1	13.8c ± 0.7	***
C18:2	66a ± 3	30a ± 9	191b ± 61	58a ± 5.2	**	3.0a ± 0.02	20.1b ± 3.7	1.8a ± 0.4	2.2a ± 0.0	***
C18:3	41a ± 3	15b ± 6	67c ± 24	26ab ± 2.8	**	1.8a ± 0.02	9.8b ± 3.0	0.6a ± 0.2	1.0a ± 0.0	***
Σ(C4:0–C8:0)	68a ± 4	28b ± 2.4	537c ± 78	47b ± 8.1	***	3.1a ± 0.1	18.8b ± 3.4	5.0c ± 0.1	1.8a ± 2.1	***
Σ(C10:0–C14:0)	410a ± 18	29b ± 2.4	1755c ± 169	530a ± 57	***	18.4a ± 0.2	19.5b ± 1.1	16.6c ± 0.3	20.6d ± 0.0	***
Σ(C16:0–C18:3)	1744a ± 99	92b ± 18.6	8308c ± 945	1998a ± 208	***	78.5a ± 0.1	61.7b ± 4.5	78.4a ± 0.1	77.6a ± 0.1	**
Σ(C4:0–C18:3)	2222a ± 126	149b ± 18.6	10600c ± 1192	2575a ± 273	***	100	100	100	100	
% total FFA[c]	14.3 ± 0.8	0.9 ± 0.1	68.2 ± 7.6	16.6 ± 1.7						

[a] The percentage of individual FFA released in each ripening room is also calculated. Values in the same row with the same letter are not significantly different according to the LSD test ($\alpha < 0.05$). [b] Probability of *F* test: ***, $P < 0.001$; **, $0.001 < P < 0.01$. [c] Considering 15546 μg g^{-1} of fat (after 8 days of storage in the 4 °C room) as maximum FFA released during ripening.

The pattern of individual FFA that were released during storage in each of the temperature-controlled rooms was modified noticeably during ripening (**Table 4**). The percentage of long-chain FFA [Σ(C16:0−C18:3)] increased from 61.7% in the 12 °C room to 78.4% in the 21 °C room. This increase was mainly due (i) to the release of palmitic acid (C16:0), which increased from 3.8% in the 12 °C room to 40.2% of total FFA in the 21 °C room, and (ii) to the release of stearic acid (C18:0) (**Table 4**). The long-chain unsaturated FA [Σ(C18:1, C18:2, C18:3) = 59.3%], myristic acid (C14:0 = 13.6%), and butyric acid (C4:0 = 12.2%) were the FFA most released in the 12 °C room (**Table 4**). The percentage of short-chain FFA [Σ(C4:0-C8:0)] detected decreased from 18.8 to 5.0% (12 °C vs 21 °C room), and the percentage of medium-chain FFA [Σ(C10:0−C14:0)] detected decreased from 19.5 to 16.6% (12 vs 21 °C room). Chamba and Perreard (*8*) previously reported an increase in the concentration of long-chain FFA in the warm room (23 °C) and a decrease in the concentration of medium- and short-chain FFA, which is consistent with the results of this study. As Chamba and Perreard (*8*) did not report any specific activity of propionic acid bacteria to the hydrolysis of long-chain FFA, it is thought that short-chain FFA may be transformed quickly by β-oxidation and/or esterification. This catabolism of FFA leads to the formation of many volatile compounds in Emmental cheese (*7*). In the 4 °C room the percentages of short-chain, medium-chain, and long-chain FFA detected were 1.8, 20.6, and 77.6%, respectively (**Table 4**). Higher levels of palmitic acid (C16:0) were released in the 21 and 4 °C rooms, respectively 40.2 and 47.4% of total FFA.

The percentage of individual FFA released compared to individual fatty acids was calculated at each stage of ripening as follows: % individual FFA released = [(FFA/FA)] × 100, and is presented in **Figure 2**. Considering their relative proportions in cheese fat, all of the FA were released due to lipolysis. Four FA were released in the 21 °C room at levels ≥1.5%, that is, C4:0, C16:0, C18:1, and C18:3, whereas the medium-chain and long-chain FFA were released at levels ≥1% (**Table 5**). All of the FA were released due to lipolysis in the

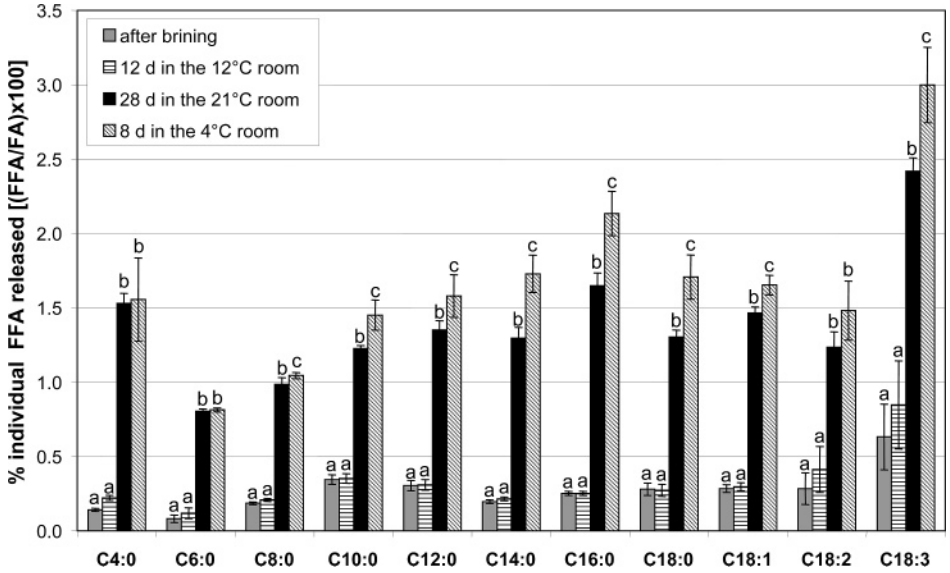

Figure 2. Percentage of individual free fatty acids compared to individual fatty acids determined at each stage of ripening of Emmental cheese. For each fatty acid, values with the same letter are not significantly different according to the LSD test ($\alpha < 0.05$).

Table 5. Percentage of Fatty Acids Released Due to Enzymatic Hydrolysis of Lipids, Obtained by Calculating the Ratio between Individual Free Fatty Acid and the Corresponding Fatty Acid after the Main Stages Involved during Ripening of Emmental Cheese[a]

	% (FFA/FA)				
fatty acid	from milk to after brining	12 days in the 12 °C room	28 days in the 21 °C room	8 days in the 4 °C room	stat[b]
C4:0	0.14ab ± 0.01	0.22ab ± 0.02	1.53e ± 0.07	1.56bc ± 0.28	***
C6:0	0.08a ± 0.03	0.12a ± 0.04	0.80a ± 0.02	0.81a ± 0.01	***
C8:0	0.19abc ± 0.01	0.21ab ± 0.01	0.98b ± 0.05	1.04a ± 0.02	***
C10:0	0.35d ± 0.03	0.35bc ± 0.03	1.23c ± 0.02	1.45b ± 0.10	***
C12:0	0.31cd ± 0.03	0.31bc ± 0.04	1.35d ± 0.06	1.58bc ± 0.14	***
C14:0	0.20abc ± 0.01	0.21ab ± 0.01	1.30cd ± 0.07	1.73c ± 0.13	***
C16:0	0.25bcd ± 0.01	0.25abc ± 0.01	1.65f ± 0.08	2.13d ± 0.15	***
C18:0	0.28cd ± 0.04	0.27abc ± 0.04	1.30cd ± 0.05	1.71bc ± 0.15	***
C18:1	0.28cd ± 0.03	0.30bc ± 0.03	1.47e ± 0.04	1.65bc ± 0.07	***
C18:2	0.28cd ± 0.11	0.41c ± 0.15	1.24c ± 0.10	1.48bc ± 0.20	***
C18:3	0.63e ± 0.22	0.85d ± 0.30	2.42g ± 0.09	3.00e ± 0.25	***

[a] Results shown are the average of three independent trials for free fatty acids and fatty acids (mean value ± standard deviation). Values in the same column with the same letter are not significantly different according to the LSD test ($\alpha < 0.05$). [b] ANOVA in the row; probability of *F* test: ***, $P < 0.001$.

4 °C room, whereas to a lesser extent for short-chain FA, particularly C6:0 and C8:0 (**Figure 3**; **Table 5**). For each stage of the ripening, C18:3 was most released compared to the concentration of this FA in cheese fat. The release of all the FA showed how nonspecific the lipolytic enzymes are during the ripening of Emmental cheese or the concomitant and complementary activity of several enzymes more or less specifically.

FFA that are released upon lipolysis of triacylglycerols during ripening make an important contribution to the characteristic flavor of Emmental cheese, especially short- and medium-chain FFA, because they have low perception thresholds. Specifically considering the short-chain fatty acids, free butanoic acid (C4:0) occurred at a greater relative concentration in cheese compared to caproic (C6:0) and caprylic (C8:0) acids, suggesting its selective release by esterases and/or lipases present in cheese or synthesis by the cheese microflora. In general, lipolytic enzymes are specific for the outer ester bonds of triacylglycerols (i.e., *sn*-1 and *sn*-3 positions). Thus, butanoic acid (C4:0) as well as the other short- and medium-chain fatty acids (C6:0−C10:0), which are located mainly at the *sn*-3 position, would be preferentially released by lipolytic enzymes. Palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1) are mainly located at the *sn*-1 position and may also be preferentially released during lipolysis (*3*). Further work is needed to identify and characterize the activities of the enzymes involved in the lipolysis of Emmental cheese.

Alteration in the Thermal Properties of Fat Due to Lipolysis. The increase in the level of total FFA during ripening decreased the initial temperature of crystallization (T_{onset}) and the final temperature of melting (T_{offset}) of fat dispersed in Emmental cheese. On cooling of the cheese, crystallization of triacylglycerols was significantly ($P < 0.001$) delayed as a function of ripening: The T_{onset} decreased from 16.47 ± 0.13 °C at the end of the 12 °C room period to 16.01 ± 0.15 °C at the end of the 21 °C room period and to 15.54 ± 0.16 °C at the end of storage in the 4 °C room. These values were significantly different according to the LSD test ($\alpha < 0.05$). The T_{offset} decreased significantly ($0.01 < P < 0.05$) during ripening. The final temperature of melting decreased from 38.86 ± 0.08 °C at the end of the 12 °C room period to 38.41 ± 0.19 °C at the end of the 21 °C room period (significantly different according to the LSD test, $\alpha < 0.05$). Then, T_{offset} decreased to 38.19 ± 0.18 °C at the end of the 4 °C room period. At the end of

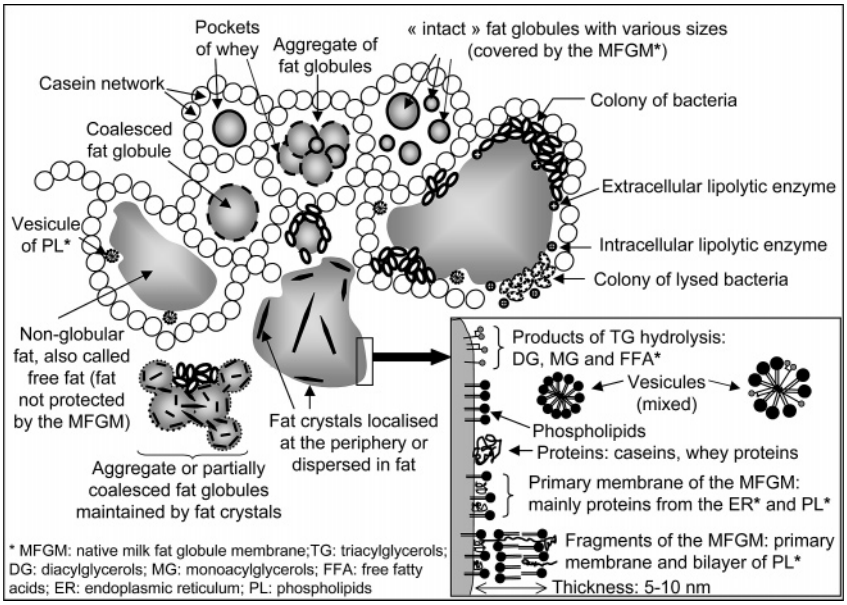

Figure 3. Schematic representation of the microstructure of Emmental cheese. The different organization structures of fat in Emmental cheese after pressing, that is, globular, aggregates, and nonglobular, are surrounded by pockets of whey entrapped in casein strands [Figure 4A, Lopez et al. (*17*)]. Fat crystals may be formed in fat inclusions at low temperature [Lopez et al. (*26*)]. Preferential localization of (lysed) bacteria is seen at the fat/protein interface and lipolytic enzymes. (Inset) Proposed composition and organization of the fat/water interface.

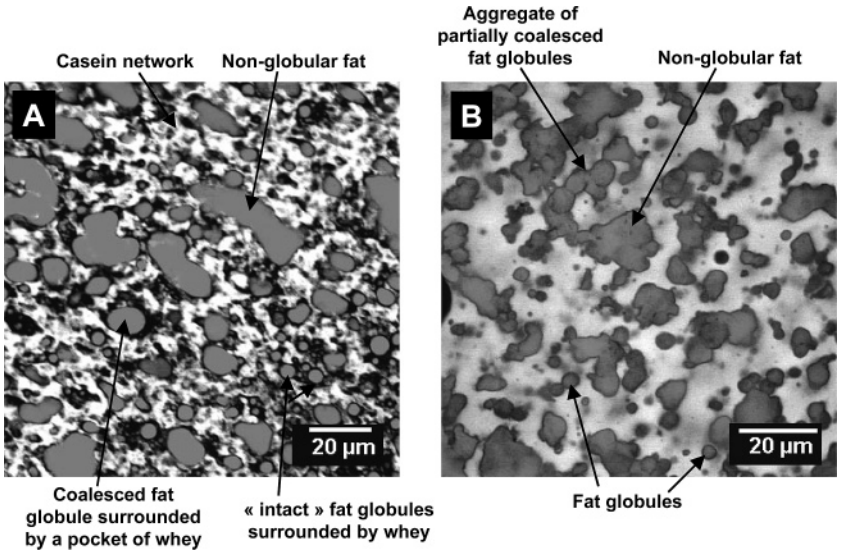

Figure 4. Confocal laser scanning micrographs taken (**A**) after pressing and (**B**) at the end of ripening of Emmental cheese. The protein network is coded in white, fat is coded in gray, and whey appears in black.

ripening, the T_{onset} and T_{offset} were not significantly different from those detected after 8 days in the 4 °C room (LSD test, $\alpha < 0.05$).

This decrease in both the T_{onset} and the T_{offset} may be related to the hydrolysis of triacylglycerols by lipolytic enzymes, as long-chain saturated fatty acids have higher melting points. Furthermore, the decrease in T_{onset} may result from the presence of phospholipids and products of lipolysis, for example, monoacylglycerols, diacylglycerols, and FFA, that may act as catalytic impurities. Although these molecules are considered to be minor components and present in cheese at low concentrations, they can affect triacylglycerol crystallization. Moreover, all of these molecules are amphiphilic compounds as they possess polar and apolar parts in their chemical structures. Thus, they may be concentrated at the fat/water interface (**Figure 3**). Recent studies have shown that crystal growth and nucleation of anhydrous milk fat are affected by the presence of partial acylglycerols, for example, diacylglycerols and monoacylglycerols, and that their interactions with triacylglycerols depend on several parameters such as the concentration of the partial acylglycerols, the fatty acid esterified, the number of fatty acids esterified, and the composition of triacylglycerols (*39*, *40*). Wright et al. (*40*) showed that the presence of minor components such as milk fat diacylglycerols delayed the onset of crystallization at low degrees of supercooling. Walstra and Jenness (*41*) suspected that micelles of monoacylglycerols act as templates for crystallization and, as such, induce heterogeneous nucleation.

Organization of Fat. Figure 4 shows the microstructure of Emmental cheese after pressing of curd grains and at the end of ripening. After pressing, fat is dispersed in the cheese matrix as (i) individual fat globules (diameter $= 0.5-10$ μm), (ii) coalesced fat globules (diameter $\approx 10-20$ μm) resulting from the fusion of fat globules with smaller size, and (iii) nonglobular fat also called free fat (inclusions $\approx 40-45$ μm) or fat not enveloped by the MFGM. Thus, processing alters the native

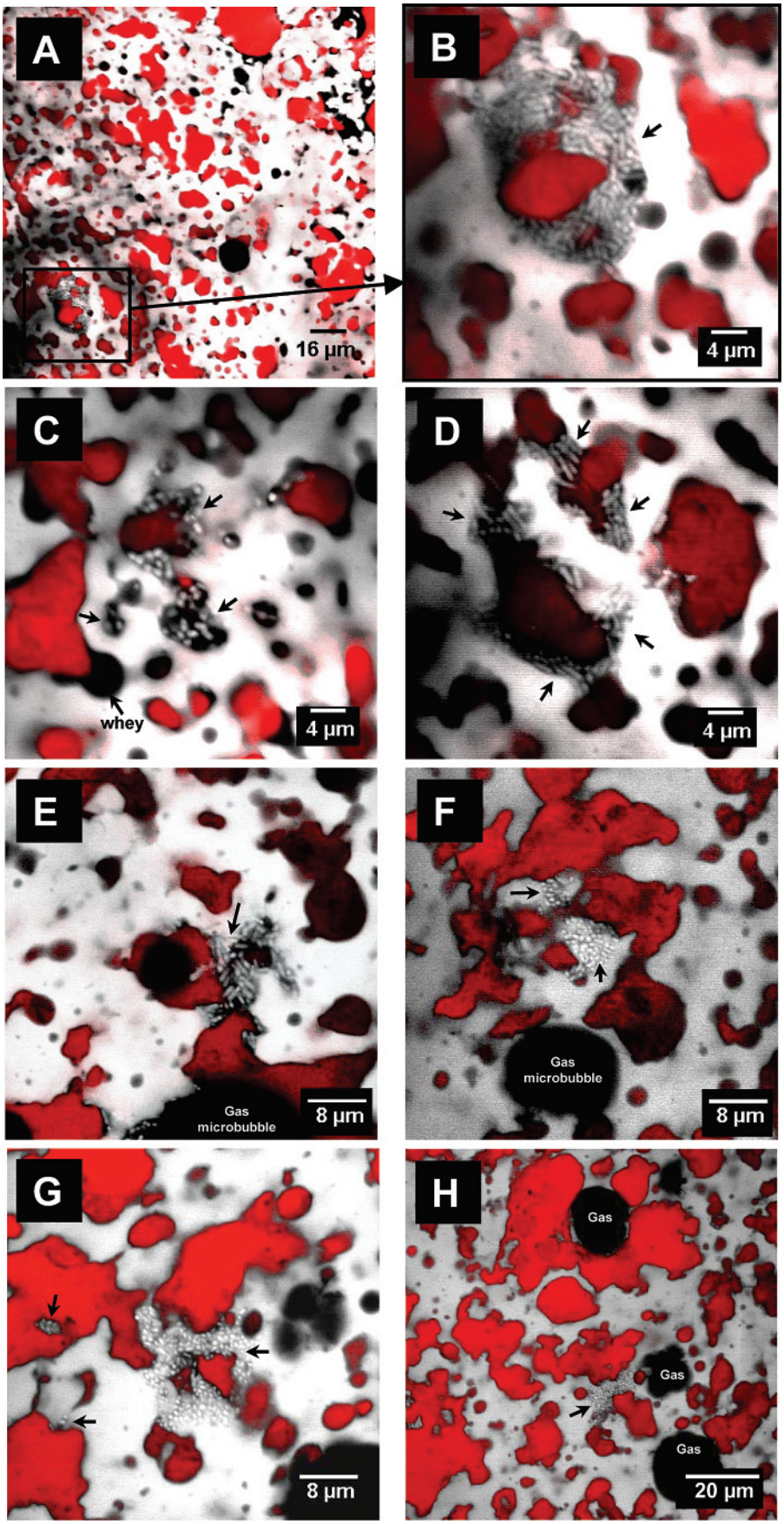

Figure 5. Confocal laser scanning micrographs taken during the ripening of Emmental cheese. The protein network and bacteria are coded in gray, fat is coded in red, and whey pockets and gas microbubbles appear in black. Localization of bacteria in Emmental cheese: (**A**) at day 1; (**B**) at day 1 with an enlarged scale permitting the visualization of a colony of bacteria; (**C**) at 1 day showing bacteria in a pocket of whey surrounding fat; (**D**) after 12 days in the 12 °C room; (**E**) after 9 days in the 21 °C room; (**F**) after 14 days in the 21 °C room; (**G**) after 8 days in the 4 °C room; (**H**) at the end of ripening. Bacteria and/or colonies are indicated by arrows.

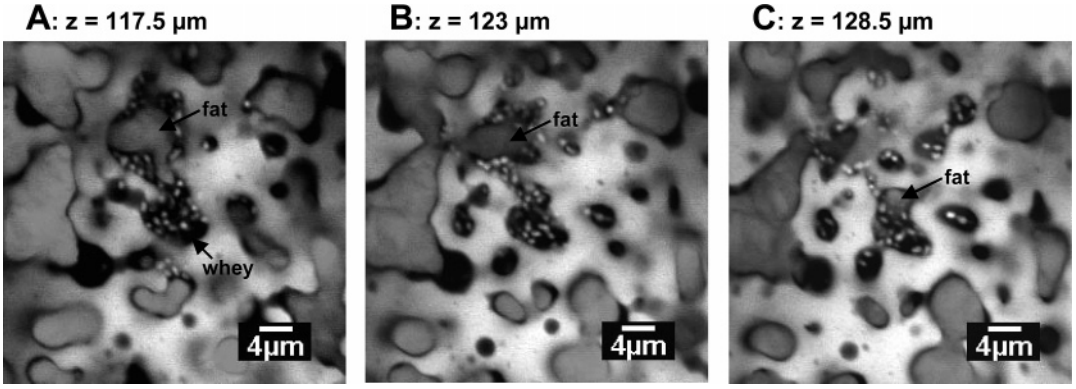

Figure 6. Confocal laser scanning micrographs taken at different depths (denoted *z*) in Emmental cheese at 1 day, showing the localization of bacteria in whey pockets surrounding fat inclusions dispersed in the protein network. The protein network and bacteria (bright areas) are coded in white, fat is coded in gray, and the whey appears in black.

milk fat globules and the native MFGM may be damaged, partially reorganized after coalescence, or disrupted (free fat). Fragments of the MFGM, the primary membrane of the native MFGM, and phospholipids may remain attached at the surface of fat (**Figure 3**). The fat/whey (aqueous) interface may also be composed of proteins (e.g., caseins, whey proteins) and products of lipolysis (e.g., FFA, monoacylglycerols, diacylglycerols) as schematically shown in **Figure 3**. Phospholipids and other amphiphilic lipids resulting from lipolysis may also form vesicles in the whey (**Figure 3**). Similar results are discussed in Lopez et al. (*17*). Depending on cheese pH, FFA may also form soaps with calcium (*42*). **Figure 4A** shows that all of the organizations of fat are surrounded by pockets of whey delimited by casein strands. At the end of ripening, fat was dispersed as (i) fat globules, (ii) aggregates of fat globules that may be partially coalesced due to the presence of fat crystals at 4 °C, and (iii) free fat (**Figure 4B**). The fat inclusions appeared to be directly surrounded by the casein network: no pockets of whey were observed around fat inclusions. Lopez et al. (*17*) showed that the supramolecular organization of fat in Emmental cheese does not change during ripening. After pressing, Emmental cheese may be considered as an oil in water emulsion with fat inclusions being the dispersed phase. At the end of ripening, the fat phase and the protein network appear to be interconnected.

Compared to the size of fat globules in cheese milk [diameter $= 4.47 \pm 0.06$ μm; Lopez et al. (*17*)], the processing steps involved in Emmental cheese manufacture (i) increased the size of fat inclusions, (ii) decreased the total fat surface area, and (iii) changed the composition of the fat globule surface by disrupting the MFGM, thereby providing a smaller fat/water interface for lipase activity.

Preferential Localization of Bacteria at the Fat/Protein Interface. Figure 5 shows bacteria in the microstructure of Emmental cheese, which grew as colonies during acidification and ripening. The first colonies of bacteria were detected at 1 day, after acidification (4 h during pressing at 47 °C, then 19 h in a temperature-controlled room at 24 °C). **Figure 5A** shows the microstructure of Emmental cheese at 1 day in which fat inclusions of various sizes were visible as well as a colony of bacteria. This colony of bacteria, localized at the fat/casein interface, is enlarged in **Figure 5B** for clarity. The colony consisted of rod-shaped bacteria, with a length of $\approx 1-2$ μm, considered to be lactobacilli that grew during acidification (*7*). **Figure 5C**, also taken at 1 day, shows bacteria mainly dispersed in the whey surrounding a fat inclusion. **Figure 6** shows micrographs taken at different depths (denoted *z*) in Emmental cheese at 1 day. The bacteria appear to be cocci (lactococci or streptococci) (*7*) and are localized in the pockets of whey. It should be kept in mind that the cheese matrix is three-dimensional, whereas the micrographs presented are only two-dimensional. The pocket of whey localized in the center of **Figure 6A** surrounded a fat inclusion, which was clearly visible in **Figure 6C** (Δdepth $= 11$ μm). Furthermore, **Figure 6** shows that some pockets of whey were connected at certain depths and separated at different depths.

Figure 5D, taken at the end of ripening in the 12 °C room, shows bacteria that grew at the fat/protein interface. Colonies of streptococci and lactobacilli have been previously observed during the ripening of Emmental cheese in the temperate room using scanning electron microscopy (SEM) (*13*). During storage of Emmental cheeses in the 21 °C room, two types of colonies were observed: bacilli and irregular cocci or short rods. The first may correspond to a colony of lactobacilli (either starter on nonstarter) (**Figure 5E**) and the second to a colony of short-rod propionic acid bacteria (bright area) (**Figure 5F**). It is known that in the 21 °C room, the temperature and growth conditions, in particular the concentration of lactate, allow the rapid growth of propionic acid bacteria (**Table 2**) (*34*). From the CLSM micrographs, an identification of the bacteria was proposed by considering their morphology characterized using SEM by Steffen et al. (*7*). At the end of the ripening period in the warm room, Rousseau and Le Gallo (*43*) did not observe colonies of propionic acid bacteria and inferred that the level of the cells was below the threshold of detection.

Figure 5G shows a colony of bacteria in the microstructure of Emmental cheese after 8 days in the 4 °C room, which could correspond to short-rod propionic acid bacteria. **Figure 5H** shows the microstructure of Emmental cheese at the end of ripening at a scale that allowed the organization of fat, the gas microbubbles (black areas), and a colony of bacteria to be clearly seen.

Figure 7 shows the three-dimensional organization of a colony of bacteria at different depths of observation (total depth $= 18.2$ μm) in Emmental cheese at the end of ripening. The observation of this colony, which had a diameter of $\approx 18-20$ μm, showed that it was in contact with both the fat and casein network in the volume of the cheese.

The microstructural analysis performed in this study using CLSM showed that bacteria were organized as colonies in Emmental cheese because individual bacteria were not observed during ripening. As acidification occurs after pressing during the manufacture of Emmental cheese, all bacteria including lactic starters grew in a structured cheese matrix (**Figure 4A**), resulting

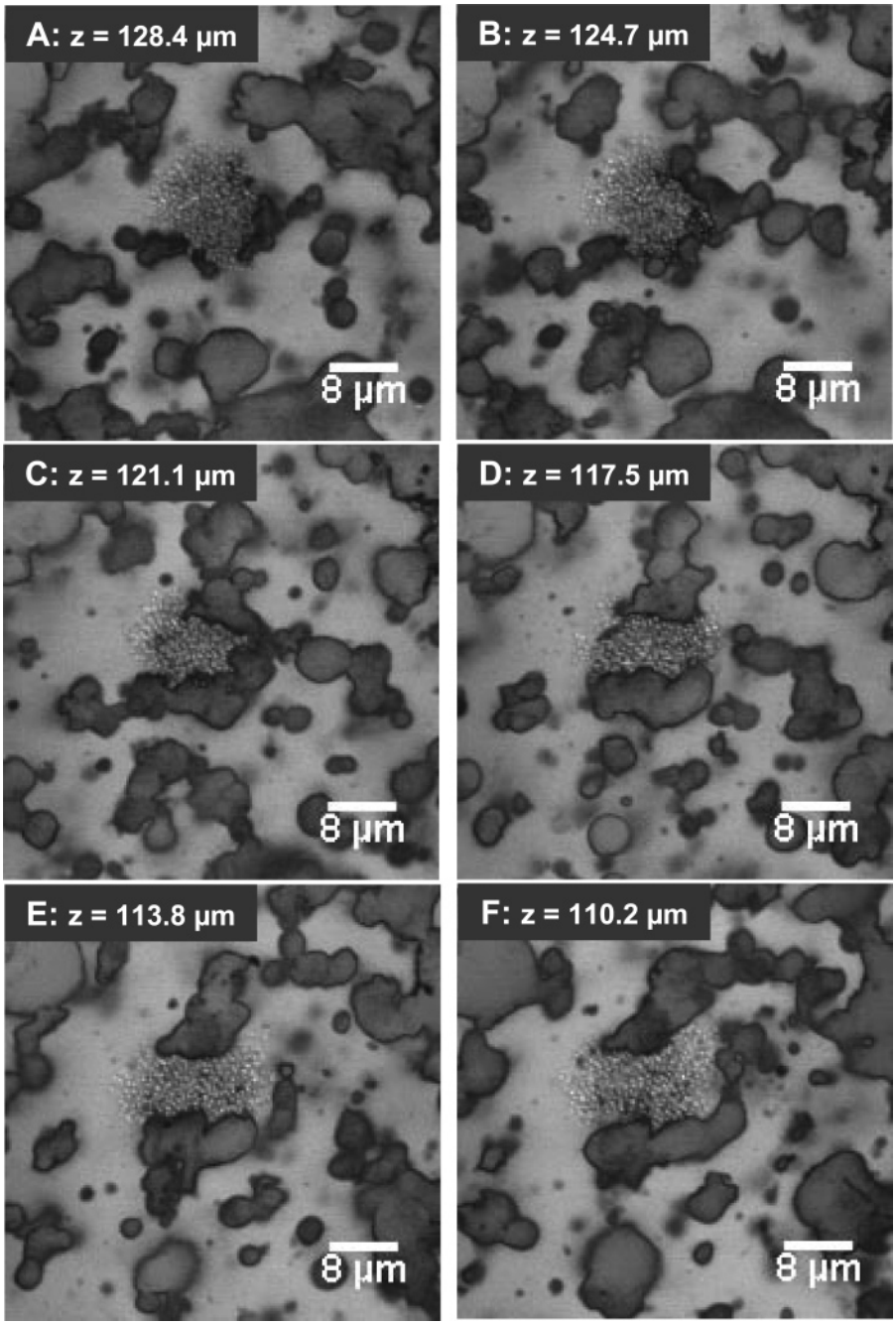

Figure 7. Confocal laser scanning micrographs taken at different depths (denoted *z*) in Emmental cheese at the end of ripening to characterize in situ the organization of a colony of bacteria. The protein network and bacteria (bright areas) are coded in white, and fat is coded in gray.

in their organization as colonies. Furthermore, this study showed that the colonies of bacteria were preferentially localized at the fat/protein interface and that both fat and bacteria were entrapped in the casein network. Similar results were obtained using SEM to characterize the microstructure of mozzarella cheese (*20*). The localization of bacteria in the cheese matrix is schematically represented in **Figure 3**.

Lipolysis Regarding Structure of Fat and Preferential Localization of Bacteria. Several authors have considered that lipolysis is essential to the flavor development and, thus, to the overall quality of Emmental cheese. Lipolysis in Emmental cheese is mainly due to the lipolytic activity of propionic acid bacteria, which is strain-dependent and not related to the production of propionic acid in cheese (*8*, *38*). Over recent years, studies on the lipolytic system of propionic acid bacteria have reported esterase and lipase activities with either an intracellular or an extracellular origin (*44*, *45*). The release of intracellular lipolytic enzymes requires lysis of bacteria as schematically shown in **Figure 3**. However, the cell lysis of propionic acid bacteria has not yet been shown. The growth of propionic acid bacteria in the 21 °C room can be related in this study to the high increase of FFA released during ripening (**Tables 2**−**4**). These results may favor an extracellular lipase activity of propionic acid bacteria because (i) lipases hydrolyze acyl ester chains of 10 or more carbon atoms, as is the case in this study (**Figure 2**), and (ii) lipases hydrolyze emulsified substrates. Furthermore, lipases are active only in the presence of a hydrophobic/hydrophilic interface. Other lipolytic enzymes such as lipoprotein lipase or esterases of lactic acid bacteria (thermophilic streptococci, thermophilic lactobacilli) can have a weak contribution to the lipolysis of Emmental cheese (*8*). It has been reported that propionic acid bacteria are between 10 and 100

times more lipolytic compared to lactic acid bacteria (*46*). However, the interaction between propionic acid bacteria and lactic acid bacteria regarding lipolysis has been reported (*38*), and the contribution of lactic acid bacteria to propionic acid fermentation is now well-known (*47*). Holland et al. (*48*) showed that esterases of lactic acid bacteria prefer di- and monoacylglycerols for both hydrolysis and ester synthesis and that they can affect both the lipolytic and ester flavors of cheese. Thus, di- and monoacylglycerols produced in the 21 °C room as a result of the hydrolysis of triacylglycerols by propionic acid bacteria may be the substrate of the lipolytic enzymes of lactic acid bacteria during storage in both the 21 and 4 °C rooms.

As highlighted by Collins et al. (*9*), previous studies on lipolysis in Emmental cheese have not considered the supramolecular organization of fat, the localization of the colonies of bacteria, and thus the accessibility of fat by bacteria and lipolytic enzymes.

In this study, which was performed using CLSM, the bacteria were for the first time shown to be organized as colonies in the matrix of Emmental cheese. Furthermore, this study showed that the colonies were preferentially localized at the fat/protein interface. This localization may be explained as follows: During the formation of the rennet-induced casein network, bacteria may be forced out of the protein matrix and thus expelled in pockets of whey surrounding fat inclusions (**Figures 4A** and **6**), where they grew and became entrapped at the fat/protein interface. Moreover, the results showed that fat globules were mainly disrupted after pressing and that fat was dispersed in the casein network as coalesced fat globules and free fat. Disruption of fat globules induced a reduction in the interfacial surface for lipase activity compared to native milk fat globules. However, the release of the MFGM, which acts as a physical barrier protecting fat against lipolysis (*41*), may facilitate the accessiblity of lipolytic enzymes to triacylglycerols.

As fat is not protected by the MFGM, the enzymatic pathways of bacterial strains normally required to hydrolyze the phospholipid−protein complex of the MFGM are not necessary. Hydrolysis of the MFGM could also be due to other microorganisms such as NSLAB that can survive after thermization.

Lipolysis is limited to triacylglycerols that are accessible to lipolytic enzymes. As these enzymes are mainly of bacterial origin and the diffusion of enzymes is limited in the protein matrix, lipolysis may be favored in fat inclusions neighboring bacteria. Thus, lipolysis may be heterogeneous in the cheese matrix and controlled by the number of bacterial colonies in the body of the cheese and by the activity of their lipolytic enzymes. Whereas Rousseau and Le Gallo (*43*) reported that the proteolysis of caseins in Emmental cheese is localized in a limited area around bacterial cells, the microheterogeneity of lipolysis has not yet been considered.

The physical state of the substrate for lipolysis, for example, triacylglycerols crystallized or liquid, may influence its potential to be attacked by lipolytic enzymes. However, crystallization of triacylglycerols may decrease the rate of hydrolysis of fatty acids. Lopez et al. (*26*) have reported that 44.08 ± 5.73% of fat was crystallized at the end of the 12 °C room period, 18.76 ± 2.03% was crystallized at the end of the 21 °C room period, and 53−55% was crystallized in the 4 °C room under time−temperature conditions of ripening similar to those used in this study. Long-chain saturated FA (stearic and palmitic acids) that have higher melting points are hydrolyzed throughout the ripening, including in the 4 °C room. Thus, lipolysis is not restricted as a result of FA crystallization, which mainly occurs at 4 °C. Storage of Emmental cheese at low temperature (12 and 4 °C) can increase the rigidity of fat, which depends on the ratio of solid to liquid fat. Fat crystals coexist with liquid triacylglycerols in fat globules and fat inclusions. The localization of fat crystals in a complex matrix such as cheese is not known. However, some authors have shown that crystals formed by high-melting triacylglycerols are located at the periphery of fat globules (*49*, *50*). Thus, these crystallized triacylglycerols may constitute a barrier for enzymes to access liquid triacylglycerols, preferentially localized in the center of fat globules (**Figure 3**).

Disruption of fat globules and damage and/or release of the protective MFGM may facilitate the reactions that generate flavor precursors and components. It has been reported that fat plays an essential chemical role in facilitating flavor generation by providing a fat/protein interface for flavor-producing reactions (*51*). Moreover, another mechanism by which fat contributes to the perception of flavor is through its capacity to dissolve flavor compounds. The majority of flavor compounds are at least partially soluble in fat, and hence fat inclusions dispersed in the casein matrix may act as a reservoir for flavor compounds, which are released when the cheese is eaten (*52*). Thus, the surface area and composition of the fat/protein interfacial region can affect flavor generation and flavor perception.

In conclusion, this work contributes to a better understanding of the mechanisms of lipolysis and, more particularly, the accessiblity of lipolytic enzymes to their substrates, which may improve the quality of Emmental cheese. The preferential localization of colonies of bacteria is shown to be at the fat/protein interface. Furthermore, analysis of the microstructure showed that fat is mainly present in the casein matrix as aggregates and free fat, which is not protected by the MFGM. Both the localization of bacteria and the composition of the newly formed fat/whey interface may facilitate the hydrolysis of triacylglycerols by lipolytic enzymes. From a methodological point of view, this study confirms that confocal laser scanning microscopy is a useful technique for the direct observation of bacterial colonies in a complex matrix, such as cheese. The formation of the structure of the cheese matrix during manufacture, that is, the organization of the protein network, of fat and the pockets of whey in which the bacteria may grow, which results from the physical (cooking, cutting, pressing) and enzymatic (rennet) treatments applied, determines the conditions of ripening, that is, localization of bacteria and accessibility to their substrate.

ACKNOWLEDGMENT

We acknowledge M.-N. Madec for microbiological analysis, E. Beaucher for physicochemical analysis, J.-Y. Gassi for help in the manufacture of Emmental cheese, R. Primault (Microscopy Department, University Rennes I) for technical assistance in confocal laser scanning microscopy, and M.-H. Famelart for help in statistical analysis. We thank A. Thierry for comments regarding the manuscript and valuable discussions on lipolysis in Emmental cheese.

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Received for review January 24, 2006. Revised manuscript received May 30, 2006. Accepted June 8, 2006.

JF060214L