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Ethyl Ester Formation Is Enhanced by Ethanol Addition in Mini Swiss Cheese with and without Added Propionibacteria

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Esters are important contributors to cheese flavor, but their mechanisms of synthesis in cheese are largely unknown. This study aimed to determine whether ethanol concentration limits the formation of ethyl esters in cheese. Mini Swiss cheeses were manufactured with (E) or without (C) the addition of ethanol to cheese milk. Ethanol concentrations (enzymatic analysis) were 64 ± 17 and 330 ± 82 μg g^{-1}, respectively, in C and E cheeses. E cheeses also contained 5.4 ± 2.3 times more of the five ethyl esters quantified than C cheeses, regardless of the concentrations of esters in C cheeses (range 1−128 ng g^{-1}). Furthermore, the presence of propionibacteria added as acid-producing secondary starters was associated with greater concentrations of esters, due to the increase in acid concentrations that propionibacteria induced and/or to an involvement of propionibacteria enzymes in ester synthesis. This study demonstrates that ethanol is the limiting factor of ethyl ester synthesis in Swiss cheese.

KEYWORDS: Ester; Swiss cheese; ethanol; propionibacteria

INTRODUCTION

Esters are key components in fruit flavor, but they also carry fruity flavor notes in alcoholic beverages, fermented dairy products, and fermented meat and vegetables, where they are commonly detected in the volatile fraction (*1*). Regarding cheeses, esters are important contributors to the flavor of soft cheeses, Italian and Swiss type cheeses, and some other cheeses (*2*), in balance with many other volatile compounds formed during cheese maturation (*3*, *4*). Esters can contribute positively to cheese flavor or be considered as a flavor defect, depending on their concentration and on the cheese variety (*2*). For example, esters are thought to contribute positively to the fruity note in Swiss cheese (*5*−*7*). However, excessive levels of ethyl esters of fatty acids of C4−C10 cause a fruity flavor defect in Cheddar cheese (*8*). The concentrations of esters dramatically vary between different individual cheeses of the same variety. For example, factors of variation of the concentration of esters up to 10−40 were observed in Swiss cheeses and in Gruyère de Comté cheese, a variety of Swiss type cheese (*6*, *9*), and up to 250-fold in two Gouda cheeses of comparable age (*10*).

Several biochemical pathways can be involved in ester formation. Esters can be formed by an esterification reaction, involving an acid and an alcohol, or by the reaction of another ester with either an alcohol (alcoholysis) or with an acid (acidolysis) or by the reaction of two esters (transesterification) (*2*). These reactions can occur spontaneously or be catalyzed by esterases (carboxyl ester hydrolases, EC 3.1.1.1), aryl esterases (aryl ester hydrolases, EC 3.1.1.2), and lipases (triacylglycerol hydrolases, EC 3.1.1.3). Esters can also result from the linkage of an acyl coenzyme A (CoA) to an alcohol, a reaction catalyzed by alcohol acyl transferases (EC 2.3.1.84). This latter reaction occurs in fruits and in alcoholic beverages by the activity of the alcohol acyl transferases of plant and yeasts, respectively (*1*, *11*).

The mechanism of synthesis of esters in cheese is still largely unknown (*2*). It is generally accepted that the enzymes of cheese microflora are involved in the formation of cheese esters (*1*, *2*), but some authors also suggested that cheese esters are not of enzymatic origin (*12*, *13*). Most S-methyl thioesters can be formed spontaneously in cheese from the reaction of acylCoA with methanethiol (*14*). Two ester-producing reactions could be involved in ester formation in cheese, esterification and alcoholysis. Until recently, ester synthesis in cheese was regarded as resulting from the esterification of an alcohol and an acid. The acid or acyl CoA moieties of esters are formed from the action of the cheese microflora and their enzymes on lactose, lactate, lipids, and proteins of cheese curd (*4*). It has been recently shown that cheese esters could also be synthesized directly from glycerides and alcohols via an alcoholysis reaction. Esterases of lactic acid bacteria can catalyze this reaction, consisting in the transfer of a fatty acyl group from triglycerides (and, preferably, mono- and diglycerides) to an alcohol, without the direct involvement of water (*15*, *16*). Alcoholysis could be a more common route of esters synthesis in aqueous environ-

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ments than esterification reaction, which is favored under low water activity conditions (*2*).

The rate-limiting factors of ester synthesis in cheese are unknown. Substrates, enzymes, and environment may all determine the rate of ester formation. In Cheddar cheese, however, ethanol is regarded as the limiting factor of ester synthesis (*2*). The "fruitiness" defect of Cheddar cheese, which results from the formation of ethyl esters (*8*), was found significantly correlated to the concentration of ethanol (*17*). It was reported that the addition of ethanol to Cheddar curd produced increased levels of ethyl esters but without giving any experimental details (*18*). Considering some published quantitative data on cheese volatiles, it could be assumed that there is a relationship between the concentration of ethanol and the concentration of ethyl esters in different cheese varieties, including Swiss cheese (*6*). Ethanol is the main alcohol detected in cheese (*19*). Its formation in cheese mainly results from the activity of heterofermentative lactobacilli and/or yeasts.

The aim of the present study was to determine whether the concentration of ethanol in Swiss cheese limits the formation of ethyl esters. In this aim, ethanol was added to cheese milk used to manufacture mini Swiss cheeses, and the concentrations of alcohols, acids, and esters were determined at two ripening stages. Because it is known that propionibacteria (PAB) strains affect the production of acids derived from fermentation, lipolysis, and amino acid catabolism (*20*), several strains of PAB and a control cheese without PAB were used to induce variations in acid concentrations.

MATERIALS AND METHODS

Cheese Manufacture. Small scale (1/100) experimental Swiss cheeses were manufactured from thermized and microfiltered milk according to a standardized cheese-making process previously described (*20*, *21*). Cheeses were manufactured according to a factorial experimental design where two factors, addition of ethanol and use of propionic cultures, were studied. Two levels of ethanol (addition or no addition) were combined with three PAB strains (*Propionibacterium freudenreichii* ITGP14, ITGP17, and ITGP23) from the collection of the Institut Technique Français du Fromage (ITFF, Rennes, France) and one control cheese without PAB, resulting in eight combinations. Lactic starters were LH100 [a mixture of strains of *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis*, obtained from Rhodia-Food (Dangé Saint-Romain, France)] and *Streptococcus thermophilus* ITGST82 and ITGST87 (from the collection of the ITFF). The amount of ethanol (99.8%, VWR Prolabo, Fontenay sous Bois, France) added in cheese milk was adjusted to 400 μg g^{-1}, after preliminary experiments in which the residual concentrations of ethanol in curd had been determined.

Two batches of each of the eight cheeses (800 g) were manufactured on two different days, over a period of 5 weeks in December and January. Each cheese was brined on day one and then divided into one cheese block of 400 g and eight sectors of 50 g before being wrapped under vacuum in Cryovac BK1L film (Cryovac-Europe, Epernon, France). The cheese-ripening conditions used were similar to those of industrial cheese making, ripening at 12 °C for 21 days and then transferred to 24 °C (warm room) until approximately 80% of the initial lactic acid had been utilized, as previously reported (*20*). To achieve this, propionic fermentation was followed by the determination of the increase in volume of the 400 g cheese, obtained by immersion of the cheese in water, and of the concentrations of lactic, propionic, and acetic acids analyzed by high-performance liquid chromatography (HPLC) as described in the second section, at four times during the warm ripening. At this stage, which was achieved after 10 ± 3 days depending on the cheeses, two sectors were taken from the warm room, and one was placed at 4 °C for 8 additional weeks of cold ripening and one was frozen at −80 °C until use. Cheeses were analyzed at both ripening stages (end of warm room and end of cold room) for their content in organic acids, neutral volatile compounds, and free fatty acids (FFA), as described hereafter.

Compositional Analysis. Samples of ripened cheeses were analyzed for moisture (oven drying at 103 °C), protein (Kjeldahl), fat (Heiss butyrometric method), and pH by classical methods as previously described (*20*, *21*). Cheese samples were thawed and cut into cubes (2.5 mm × 2.5 mm × 2.5 mm), which were mixed. A cheese homogenate was prepared for the determination of carboxylic acids, ethanol, and neutral volatile compounds by mixing a 10 g sample with 50 g of boiled deionized water for 4 min at 20500 rpm using an Ultraturrax blender (Janke & Kunkel, Staufen, Germany). For analysis of carboxylic acids, homogenates were incubated for 1 h at 40 °C, prior to centrifugation at 8000*g* for 30 min. The supernatant was then filtered on Whatman 40 filters and diluted with the same volume of 0.005 M H_2SO_4 before filtering on a 0.45 μm pore membrane (Whatman). Lactic, propionic, and acetic acids were determined by HPLC on an Aminex A-6 ion exchange column (Bio-Rad, Hercules, CA) at 55 °C with 0.005 M H_2SO_4 as an eluent, at a flow rate of 1.0 mL min^{-1}. Both UV (210 nm) and refractometric detectors were used.

Microbiological Analyses. For microbiological analyses, performed after 2 weeks of ripening in the warm room, 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. PAB were enumerated on lithium−glycerol agar incubated at 30 °C anaerobically for 6 days, and the presence of contaminating nonstarter lactobacilli was checked on facultative heterofermentative lactobacilli agar incubated at 37 °C anaerobically for 3 days as previously reported (*20*).

Determination of Volatile Compounds. FFA ($C_{4:0}$ to $C_{20:1}$, including $iC_{5:0}$ + $aC_{5:0}$ and conjugated linoleic acid) analyses were performed by gas chromatography (GC) by ITERG (Pessac, France) as previously described (*22*). Briefly, FFAs were extracted from cheese with diethylether/heptane (1:1, v/v) after grinding with sodium sulfate and addition of sulfuric acid, isolated from lipids using an aminopropyl column, and analyzed by GC under the following conditions: cooler on-column injector; column, QUADREX−FFAP capillary column, 30 m × 0.32 mm × 0.25 μm film thickness; carrier gas, hydrogen, 1.1 bar; temperature program, heating rate 10 °C min^{-1} from 50 °C up to 240 °C, maintained for 15 min; and flame ionization detector operated at 260 °C. Acetic and propionic acids were extracted from cheese by a 50−50 mixture (v/v) of diethyl ether and petroleum ether at acidic pH, addition of ethanol 80% and NaOH, decantation, and evaporation of the hydroalcoholic phase containing the sodium salts of fatty acids, which were stored at −18 °C until analysis by GC, as previously described (*23*).

Neutral volatile compounds, including ethanol, were analyzed in the cheese homogenates described above. For ethanol determination, homogenates were centrifuged at 8000*g* for 30 min at 4 °C, and the supernatants were analyzed by the enzymatic method through the use of Boehringer kits. The other neutral volatile compounds were identified and quantified by dynamic head space GC-MS. A 7 g sample of cheese homogenate (±0.05 g) was used for each head space GC-MS analysis. Each sample was analyzed in duplicate. Briefly, volatile compounds were trapped on a Vocarb 3000 trap (Supelco, Bellefonte, PA), thermally desorbed at 250 °C, and cryofocused at −100 °C, before being injected into a HP5890 (Agilent Technologies, Palo Alto, CA) gas chromatograph−HP5972A quadrupole mass spectrometer (GC-MS). Volatiles were separated on a HP5 capillary column (60 m × 0.32 mm × 1.0 m film thickness) under the following conditions: carrier gas, helium, 29 cm s^{-1} at 35 °C; temperature program, 35 °C for 5 min, heating rate 5 °C min^{-1} up to 140 °C and then 15 °C min^{-1} up to 250 °C. MS was operated in the scan mode within a mass range of *m/z* 25−173 at 4.83 scan s^{-1}, after ionization by electronic impact at 70 eV, as described in detail previously (*24*).

Ten esters were identified, six by comparison of mass spectra and retention times with those of authentic standards (ethyl acetate, >99.9% purity, purchased from Fluka, ethyl propionate, ethyl butanoate, ethyl hexanoate, and propyl propionate, all at 99% purity, and 3-methylbutyl propionate 98% purity, all purchased from Aldrich), and the four remaining (methyl propionate, butyl acetate, isopropyl propionate, and ethyl octanoate), for which standards were not available, were tentatively

Table 1. Effect of the Addition of Ethanol and the Presence of *P. freudenreichii* (P) on the Concentrations of Short Chain Fatty Acids and Their Ethyl Esters in Mini Swiss Cheeses

compound	ripening stage[a]	without ethanol added		with ethanol added		level of significance[d]		
		without P[b]	with P[c]	without P[b]	with P[c]	E	P	E × P
ethanol (μg g^{-1})	WR	53a	66a ± 19	264b ± 34	353b ± 83	***	NS	NS
				short chain fatty acids (μg g^{-1})				
acetic acid	WR	335a ± 229	2330b ± 341	311a ± 16	2310b ± 202	NS	***	NS
	rip	477a ± 44	2375b ± 636	406a ± 128	2460b ± 147	NS	***	NS
propionic acid	WR	5a ± 6	6028b ± 978	2a ± 1	6344b ± 642	NS	***	NS
	rip	11a ± 4.2	6475b ± 1561	6a ± 6.4	7279b ± 259	NS	***	NS
butanoic acid	WR	14.5a ± 3.5	29.3b ± 4.1	11.5a ± 2.1	28.8b ± 1.9	NS	***	NS
	rip	18.5a ± 0.7	47.3b ± 5.6	20.0a ± 5.7	47.3b ± 5.8	NS	***	NS
hexanoic acid	WR	5.5a ± 2.1	21.5b ± 5.0	5.5a ± 0.7	21.3b ± 2.7	NS	***	NS
	rip	7.5a ± 0.7	28.7b ± 3.4	9.0a ± 2.8	28.8b ± 1.9	NS	***	NS
octanoic acid	WR	8.0a ± 1.4	21.5b ± 5.1	8.5a ± 2.1	20.5b ± 2.9	NS	***	NS
	rip	8.5a ± 0.7	26.5b ± 4.5	9.0a ± 0.0	25.5b ± 3.7	NS	***	NS
				main esters (ng g^{-1})[e]				
ethyl acetate (61)	WR	0.3a ± 0.1	6.7a ± 10.3	0.9a ± 1.3	4.5a ± 5.4	NS	NS	NS
	rip	0.8a ± 0.4	1.0a ± 0.8	2.6b ± 1.5	4.7c ± 0.7	***	*	*
methyl propionate #	WR	0.0a ± 0.0	2.5b ± 0.73	0.0a ± 0.0	2.4b ± 0.9	NS	***	NS
	rip	0.0a ± 0.0	11.8b ± 2.6	0.0a ± 0.0	10.0b ± 1.8	NS	***	NS
ethyl propionate (TIC)	WR	0.0a ± 0.0	22.2a ± 7.5	0.5a ± 0.7	194.4b ± 69.6	**	***	**
	rip	0.4a ± 0.4	128.4b ± 59.1	1.8a ± 1.1	652.7c ± 46.3	***	***	***
ethyl butanoate (88)	WR	5.4a ± 0.9	2.5a ± 1.7	31.7c ± 21.2	19.6b ± 3.4	*	NS	NS
	rip	12.8a ± 8.6	6.7a ± 4.4	46.7b ± 30.8	44.1b ± 6.2	***	NS	NS
ethyl hexanoate (88)	WR	3.5a ± 0.2	2.6a ± 1.8	18.7a ± 9.5	23.7 ± 9.6	*	NS	NS
	rip	10.7a ± 7.6	11.4a ± 7.0	27.5ab ± 16.1	80.0b ± 20.7	***	**	**
ethyl octanoate #$ (TIC)	WR	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.13 ± 0.10	NS	NS	NS
	rip	0.13a ± 0.13	0.09a ± 0.23	0.17a ± 0.10	0.68b ± 0.23	**	*	**

[a] WR, at the end of the warm room period; rip, ripened. [b] Results are means ± standard deviation of duplicate cheeses. Values in the same row at the same ripening stage with the same letter were not significantly different according to the LSD test ($\alpha < 0.05$). [c] Results are means ± standard deviation of six experiments (averaged values across three PAB strains and duplicates). Values in the same row at the same ripening stage with the same letter were not significantly different according to the LSD test ($\alpha < 0.05$). [d] Results of the analysis of variance: E, factor ethanol; P, factor PAB. E × P, interaction between the two factors. Probability of *F* test: ***$P < 0.001$; **$0.001 < P < 0.01$; *$0.01 < P < 0.05$; and NS, $P > 0.05$. [e] Quantification ions: TIC, total ion current peak areas or a selected fragment as indicated; #, compounds identified by comparison of mass spectral data with those of NIST 75K database; $, expressed as arbitrary units.

identified on the basis of mass spectral data from the Hewlett-Packard Chemstation NIST 75K mass spectral Database. The four main ethyl esters (ethyl acetate, ethyl propionate, ethyl butanoate, and ethyl hexanoate) were quantified from the regression curve of the corresponding standard, using external standard calibration, as previously described (*20*). Briefly, peaks were quantified by the areas of either the total ion current (TIC) or selected fragments (*m/z*). An aliquot of a mix of standard compounds was accurately weighted and used to spike a 35 g sample of control cheese homogenate, resulting in final concentrations of 2200 μg g^{-1}. Seven additional calibration standard solutions were prepared by further dilution (weight to weight) of the spiked cheese homogenate in blank cheese homogenate, in order to obtain eight different concentrations covering the range 17−2200 ng g^{-1}.

Statistical Analyses. Concentration data of each compound of duplicate cheeses were used for statistical analysis. Analyses of variance (ANOVA) were performed using the General Linear Model procedure of Statgraphics Plus (Statistical Graphic Corp., Englewood Cliffs, NJ) to determine the effects of ethanol addition, PAB presence, and the effect of the interaction between ethanol and PAB on the concentration of each volatile compound. ANOVA were also performed to determine the effects of ethanol addition, PAB strain, and the effect of the interaction between ethanol and PAB strain on the concentration of each volatile compound. Differences between the treatment means were compared at the 5% level of significance using the Fisher's least significance difference (LSD) test.

RESULTS

Cheese Gross Composition and Microbiology. The rough composition of the cheeses (62.1 ± 0.6% total solids, 45.6 ± 0.6% fat in dry matter, and 53.0 ± 0.6% moisture in the nonfat substance) was consistent with the expected values for this type of mini cheeses (*20*) and was not influenced by the addition of ethanol in cheese milk (data not shown). Nonstarter lactic acid bacteria, enumerated on two different media in cheeses after 2 weeks of ripening in the warm room, reached populations lower than 10^5 colony-forming units (cfu) per g cheese, with 50% of the cheeses below 10 cfu per g, irrespective of the addition of ethanol and of the presence of PAB. PAB numbers ranged from 3×10^9 to 4×10^9 cfu g^{-1} after 14 days in the warm room in cheeses inoculated by PAB, whereas indigenous PAB ranged between 5×10^3 and 2×10^5 cfu g^{-1} in cheeses that were not inoculated by PAB.

Effect of Ethanol and PAB on Ester Concentrations. Ethanol concentrations showed mean values of 64 ± 17 and 330 ± 82 μg g^{-1}, respectively, in the control cheeses and in the cheeses with added ethanol. Ethanol concentrations were not affected by the presence of PAB (**Table 1**) and did not significantly vary during the ripening period (data not shown).

Ten esters were identified in cheeses. The concentrations of the four main ethyl esters varied over a large range, from 1−5 (for ethyl acetate) to 128−653 ng g^{-1} (for ethyl propionate), in cheeses containing PAB, whereas ethyl butanoate and ethyl hexanoate had intermediary concentrations (7−80 ng g^{-1}). Ethyl propionate was by far the most abundant ester, as previously reported in mini Swiss cheese (*20*). Methyl propionate concentrations ranged from 2 to 12 ng g^{-1}. The concentrations of the five ethyl esters, of methyl propionate, of ethanol, and of short chain acids and the results of ANOVA are given in **Table 1**. The four other esters detected (isopropyl propionate, propyl propionate, 3-methylbutyl propionate, and butyl acetate) were detected in trace amounts.

The addition of ethanol in cheese milk resulted in significant increases in the concentrations of ethyl acetate (in ripened

cheeses only), ethyl propionate, ethyl butanoate, ethyl hexanoate, and ethyl octanoate. More precisely, the 5-fold increase in ethanol concentration induced a 3.8 ± 0.6-fold increase in the concentrations of ethyl esters in the cheeses with ethanol added, as compared with the control cheeses, in the absence of PAB. In the presence of PAB, the addition of ethanol induced a 7.1 ± 0.6-fold increase in the concentrations of ethyl esters. The concentrations of ethyl acetate at the end of the warm room period showed unexpectedly high values and variability, as compared to the ones observed in ripened cheeses, due to the high values observed in two of the cheeses (those inoculated with one of the three PAB strains used, ITGP23). The concentrations of methyl propionate were unaffected by the addition of ethanol.

The presence of PAB had a significant effect on the concentrations of all short chain acids and also influenced the concentrations of some esters, as expected (**Table 1**). Propionic acid, which results from the fermentation of lactic acid by PAB, reached concentrations ~1000-fold greater in cheeses containing PAB, as compared to cheeses without PAB. Acetic acid concentrations were 5−7-fold greater, and the concentrations of short chain acids derived from lipolysis (butanoic, hexanoic, and octanoic acids) increased by a factor of 2−4, in the presence of PAB. The presence of PAB also influenced the concentrations of some esters (**Table 1**). However, there was not a direct relationship between the concentrations of acids and the corresponding esters. Regarding esters of propionic acid, since only minute amounts of this acid were detected in cheeses without added PAB, the esters of propionate, as a direct consequence, were either undetected (methyl propionate) or present at very low concentrations (ethyl propionate) in these cheeses. The presence of PAB induced a significant increase in the concentrations of ethyl acetate, ethyl hexanoate, and ethyl octanoate at the end of ripening only in the cheeses with added ethanol, as shown by the statistical interactions observed between the factors ethanol and PAB. On the contrary, the presence of added PAB was not associated with an increase in concentration of ethyl butanoate and ethyl hexanoate at the end of the warm room period, despite the increase in concentrations of the corresponding acids, butanoic acid (×~2.5), and hexanoic acid (×3.9) (**Figure 1**).

The strain of PAB used slightly affected the concentrations of short chain acids derived from lipolysis. Hence, the concentrations of butanoic and hexanoic acid were 26 and 17% greater, respectively, in the cheeses containing strain ITGP23 than in the ones containing ITGP14. Regarding the concentrations of the corresponding ethyl esters, the strain of PAB did not significantly affect the concentrations of ethyl butanoate and ethyl hexanoate in the control cheeses (without ethanol). In the cheeses added with ethanol, PAB strain significantly ($P = 0.02$) affected only the concentrations of ethyl hexanoate, which were 28 and 61% greater, in the cheeses with strain ITGP14 and ITGP17, respectively (**Figure 1**). Therefore, no general conclusion can be drawn about the effect of PAB strain on ester synthesis. The experimental ripening procedures were designed to obtain a similar degree of propionic fermentation; therefore, the concentrations of acetic acid and propionic acid did not vary as a function of PAB strains. PAB strains had no significant effect on the concentrations of corresponding esters, ethyl acetate and ethyl propionate, and methyl propionate (data not shown).

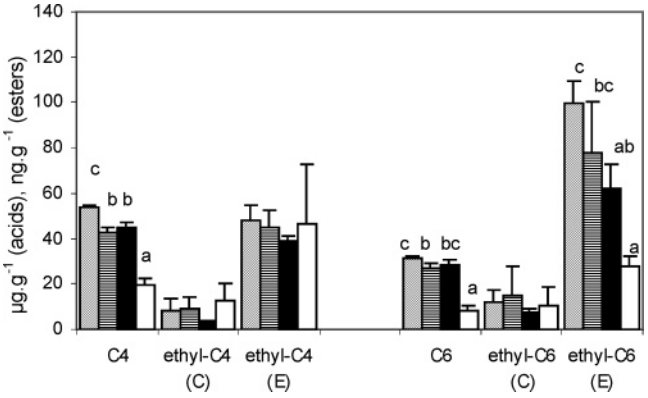

Figure 1. Effect of PAB strains and ethanol addition on the concentrations of ethyl butanoate (ethyl-C4) and ethyl hexanoate (ethyl-C6), expressed in ng g^{-1}, in mini Swiss cheeses at the end of ripening. Ethanol was added (E) or not in cheese milk (C). The concentrations of butanoic acid (C4) and hexanoic acid (C6) are also shown (μg g^{-1}). The PAB strains were as follows: ITGP23, thin hatched bars; ITGP14, horizontal hatched bars; ITGP17, solid bars; and no PAB added, white bars. Bars and error bars show the means and the standard deviation of duplicate cheese batches for esters and four cheese batches (duplicate × two ethanol levels) for acids. Bars with a different letter were significantly different according to the LSD test ($\alpha < 0.05$).

DISCUSSION

The addition of ethanol in cheese induced an increase in the concentrations of all ethyl esters. Interestingly, ethyl ester concentrations varied by a similar factor (~5) to ethanol concentration, between cheeses with and without added ethanol, regardless of the concentrations of each ester. For example, the addition of ethanol induced an increase in ethyl hexanoate concentration from 6.7 to 44 ng g^{-1} and an increase in ethyl propionate concentration from 128 to 652 ng g^{-1}. This result demonstrates that ethanol is the limiting factor of ethyl ester synthesis in Swiss cheese. Similar results were observed in Cheddar cheeses containing high levels of ethanol, either added (*18*) or which resulted from the activity of indigenous microflora (*17*). More generally, data on the volatiles in various cheeses suggest that the concentrations of esters seemed to be directly related to the concentrations of the corresponding alcohols, which suggests that alcohol concentrations could limit ester synthesis in various types of cheese. For example, two samples of Swiss cheese that showed concentrations of ethyl butanoate 5−10-fold higher than the four other samples analyzed also contained 2−10-fold higher concentrations of ethanol (*6*). Similarly, in Ras cheese, ethyl propionate and ethyl butanoate were found 10−90 and ~5 times more concentrated, respectively, in a sample that contained 2−9 times more ethanol (*25*). In Feta type cheese, two branched chain esters derived from 3-methylbutanol were detected only in the cheeses that contained the highest levels of 3-methylbutanol (*26*). The concentrations of ethanol in cheese vary over large ranges, i.e., 3−230 μg g^{-1} in Swiss cheese (*27*, *28*) and 0−620 μg g^{-1} in Cheddar cheese (*27*, *29*). Ethanol, as with other alcohols, is present in greater levels in raw milk cheeses, as compared to pasteurized cheeses (*30*). Ethanol in cheese is thought to result from the activity of obligatory heterofermentative lactobacilli and/or from yeasts (*31*). As an example, the addition of obligatory heterofermentative lactobacilli (*Lactobacillus fermentum* and *L. buchneri*) as adjunct cultures to Emmental cheese induced a 2−4-fold increase in ethanol concentrations, as compared to the control cheeses (140−230 vs 60−70 μg g^{-1}) (*28*). Other alcohols are also detected at lower levels: Isobutanol, 2-methylbutanol, and 3-methylbutanol derive from the conversion of valine, isoleucine, and leucine, respectively. Methanol is also detected in various cheeses such as Swiss type cheeses, Cheddar,

Parmesan, and Ras (*9*, *17*, *24*, *25*, *27*), but the origin of methanol, to the authors' knowledge, is unknown.

The rate-limiting factors of ester synthesis in cheese are unknown, as underlined by Liu in a recent review (*2*). Under our experimental conditions, ethanol concentration was the limiting factor of ester synthesis. Moreover, we did not observe any direct relationship between the concentrations of acids and the concentrations of the corresponding esters. The same conclusion can be drawn from the study of published quantitative data on cheese volatiles in various cheeses, i.e., Gouda (*32*), Grana (*33*), and Minas (*34*). However, it is probable that over a threshold ethanol concentration, other factors, such as enzymes and/or acid levels, would limit ester synthesis. For example, in a washed-curd cheese containing high levels of ethanol due to the use of a starter system, which included the ethanol-producing species *L. fermentum*, the levels of ethyl esters formed varied by a factor of 5−10 depending on the presence of *Geotricum candidum*/*Yarrowia lipolytica* used as adjunct cultures (*35*). This increase in ester formation in these cheeses could have resulted from yeast ester-forming enzymatic activities and/or from the increase in FFA concentrations (about 10-fold increase).

The role of PAB in ester formation remains unclear. In the cheeses containing added ethanol, the presence of PAB was associated with an increase in most ethyl esters. This effect of PAB could be due either to a direct involvement of PAB enzymes in ester synthesis, to an indirect effect due to the increase in acid concentrations, or by a combination of both these factors. Because the presence of PAB was associated with the appearance or the increase in the concentration of all acids, according to previously reported results (*20*), the hypothesis that PAB could act just as providers of the acid moiety (as acid or acylCoA) cannot be excluded. The observation that some esters were present at similar concentrations in the absence and in the presence of PAB, as observed for ethyl butanoate, suggests that the formation of at least these esters was catalyzed by the enzymes of lactic acid starters and not of PAB. Specific studies are thus necessary to investigate the role of PAB enzymes in ester synthesis and the reaction involved (esterification or alcoholysis).

Ester synthesis in cheese occurred as a separated phenomenon to microbial growth, microbial fermentation, and even lipolysis. Hence, the major proportion of esters was formed during the cold storage of cheese (8 weeks at 4 °C), i.e., during a period where all the cheese microflora did not grow anymore. Therefore, ester synthesis could have been catalyzed by the enzymes either present in metabolically active cells or released after cell lysis. Is has been shown that the lysis of lactococci, which results in the release of their intracellular esterase in cheese paste, induced an increase in lipolysis in Cheddar cheese (*36*). Whether cell lysis affects or not the synthesis of esters, however, is unknown. The decoupling between propionic fermentation and ethyl propionate synthesis was very marked. Seventy-five percent of the final levels of ethyl propionate was formed during the cold-ripening period, whereas only 10% of the total level of the corresponding acid, propionic acid, was produced during this period. Regarding the decoupling between lipolysis and ester synthesis, about 50−60% of ethyl esters of butanoic and hexanoic acids was released during the cold storage period, whereas ∼30% of the corresponding acids was synthesized during this period. This result suggests that either different enzymes would be involved in FFA release and in ester formation or that temperature induces different effects on the catalysis of ester synthesis and triglyceride hydrolysis.

The concentrations of esters ranged from ∼1 to 650 ng g^{-1}. These values are of the same order of magnitude than the ones found in full size Swiss cheese (between 26 and 98 ng g^{-1} for ethyl butanoate range and between 35 and 142 ng g^{-1} for ethyl hexanoate) (*5*, *7*). The concentrations of ethanol were ∼50−350 μg g^{-1}, whereas the concentrations of the corresponding acids ranged from ∼5 to 6000 μg g^{-1}. Hence, ethyl esters were about 100−1000 times less concentrated than acids and ethanol. Even if some losses of esters during cheese ripening cannot be excluded, these values show that the balance of esterification reaction is in favor of hydrolysis rather than synthesis, likely due to the aqueous environment.

The mini cheeses manufactured in this study did not permit sensory analyses to be performed. The presence of high levels of esters in cheese is often associated with a "fruity" aroma, which can either contribute positively to cheese flavor or be considered as a flavor defect, depending on the cheese variety (*2*). Whether the increase in ester concentrations observed in this study could induce flavor changes has now to be further investigated.

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