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Varied volatile compounds are produced by *Propionibacterium freudenreichii* in Emmental cheese

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Abstract

Directing cheese flavour development requires to know the respective contribution of each of the cheese microflora. Volatile (flavour) compounds produced by *Propionibacterium freudenreichii,* the main secondary flora of Emmental cheese were identified by gas chromatography-mass spectrometry in mini Emmental cheeses, and in pure cultures of propionibacteria grown in cheese aqueous phase (juice). Selected compounds were quantified. Propionibacteria significantly influenced the amount of 57 out of the 69 volatiles identified in juice. Short-chain carboxylic acids (acetic, propionic, butanoic, hexanoic, and isovaleric acids), esters (mainly esters of acetic and propionic acids), and some ketones and alcohols were more abundant in the presence of propionibacteria, whereas most aldehydes were less abundant. Most of these volatiles were branched-chain compounds. In cheese, the fingerprint of propionibacteria on the volatile profile, despite being less pronounced, showed similar trends as in juice. Carboxylic acids and esters are the most probable cheese flavour-active compounds produced by propionibacteria.

Keywords: *Propionibacterium*; Volatile compounds; Emmental cheese; GC-MS; Quantification

1. Introduction

Cheese flavour results from a complex mixture of various volatile and non-volatile compounds. Volatile compounds mainly originate from the enzymatic degradation of cheese carbohydrates, proteins and lipids by cheese micro-organisms during cheese processing (McSweeney, Nursten, & Urbach, 1997). To control or modify cheese flavour, it is necessary to understand the role of cheese microflora in the formation of flavour compounds.

In Emmental cheese, thermophilic lactic acid bacteria and propionibacteria (PAB) are known to be essential for the development of the characteristic flavour (Langsrud & Reinbold, 1973). Thermophilic lactic acid bacteria (streptococci and lactobacilli) perform curd acidification, achieve the fermentation of the whole milk lactose to lactate within the first day of manufacture, and play a prominent role in curd proteolysis, liberating free amino acids which are precursors of flavour compounds. Lactic acid bacteria are also able to form various volatile compounds, and in particular significant amounts of carbonyl compounds (Imhof, Glattli, & Bosset, 1995; Reps, Hammond, & Glatz, 1987). PAB are known to ferment lactic acid to acetic and propionic acids (Bergère & Accolas, 1986; Hettinga & Reinbold, 1972) and were shown more recently to play a key role in the formation of free fatty acids and isovaleric acid, which respectively result from lipolysis (Chamba & Perreard, 2002), and from leucine/ isoleucine catabolism (Thierry, Richoux, & Kerjean, 2003). However, their ability to form volatile (flavour) compounds has not been fully investigated.

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The aim of this work is to determine how PAB contribute to cheese flavour, i.e. to identify the flavour compounds PAB produce and their mechanisms of formation. In this preliminary study, we make an inventory of the volatile compounds which are produced in the presence of *Propionibacterium freudenreichii* in mini-Emmental cheeses. The volatiles that this species produces in pure culture in a liquid model cheese medium (Emmental cheese juice) are identified. Selected compounds were quantified in juice and cheese to evaluate their possible roles in cheese flavour.

2. Materials and methods

2.1. Growth of PAB in Emmental cheese juice medium

The aqueous phase (cheese juice) of an Emmental cheese was used as a culture medium for *P. freudenreichii* subsp. *shermanii* TL34 from the TL collection (Laboratoire de Recherches de Technologie Laitière, INRA, Rennes, France). Juice was extracted from a cheese before it entered the warm room, by pressing a mixture of grated cheese and sand (1:2, w/w) as previously described (Salvat-Brunaud et al., 1995). It was sterilised using 0.2 μm cellulose acetate membranes (Sartorius, Palaiseau, France). PAB cells were grown in juice medium under conditions simulating Emmental cheese, as previously described (Salvat-Brunaud, Thierry, & Maubois, 1997). Juice was inoculated with 1% of a culture grown in the same medium and incubated anaerobically at 24 °C (in anaerobic jars with Anaerocult A, Merck, Nogent-sur-Marne, France). At the end of incubation, analyses were carried out for three distinct cultures. An aliquot of uninoculated juice was also incubated under the same conditions, to be used as control. PAB growth was followed by plating serial dilutions on YELA (Thierry, Maillard, & Yvon, 2002) incubated at 30 °C anaerobically for 6 days.

2.2. Cheese manufacture and ripening

Laboratory scale (1/100) Emmental cheese trials were performed by the Institut Technique Français des Fromages according to a standardised cheese-making process which was previously detailed (Buisson, Kerjean, & Courroye, 1987; Richoux, Faivre, & Kerjean, 1998; Richoux & Kerjean, 1995). Milk was thermised (65 °C, 20 s) and microfiltered before use. The lactic starters (*S. thermophilus* TA060, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis* LH100, Rhodia-Food, Dangé Saint Romain, France), and *P. freudenreichii* subsp. *shermanii* ITGP22 (collection of the Institut Technique Français du Fromage, Rennes, France) were added in assay cheese milk, whereas a control cheese was manufactured without added PAB. Milk was pre-incubated with starters at 32 °C for 1 h and then coagulated by the addition of calf rennet (Chr Hansen, Beaune, France). When the desired firmness was reached, the gel was cut into pieces of 4 mm, stirred for 15 min and cooked at 53.5 °C for 45 min. The pressing step (6 h) and the acidification step (14 h) were conducted in thermostatted ovens at 48 and 36 °C, respectively, in order to mimic the cooling rate of the centre of a full-size Emmental wheel. Cheeses were then cooled and salted in a NaCl-saturated brine at 7 °C for 3.5 h. Each cheese was manufactured in duplicate. Cheeses were divided into four pieces (200 g each) before waxing. Cheeses were ripened at 11 °C (cold room) for 21 days, at 24 °C (warm room) for 28 days, and then stored at 4 °C for 28 days. The gross composition of ripened cheeses was determined by the Institut Technique Français des Fromages by usual methods (Richoux et al., 1998).

The presence of contaminating non-starter lactic acid bacteria in experimental cheeses was checked on FH agar (Isolini, Grand, & Glättli, 1990) incubated at 37 °C anaerobically for 3 days. The growth of PAB was followed by plating serial dilutions on YELA as described for juice cultures.

2.3. Analyses of neutral volatile compounds

Neutral volatile compounds were detected by dynamic head space-GC-MS. Before analysis, cheese samples were thawed and cut into cubes (2.5 mm × 2.5 mm × 2.5 mm) which were mixed. A 15 g sample was homogenised with 60 g of 0.5 M sodium citrate solution by mixing 4 min at 20,500 rpm using an Ultraturrax blender (Janke & Kunkel, Staufen, Germany). A 7 g sample of cheese homogenisate or a 4 g sample of cheese juice (±0.05 g) was used for each head space-GC-MS analysis. Each sample was analysed in duplicate (cheese) or in triplicate (juice). Analyses were carried out as described in detail elsewhere (Thierry, Maillard, & Le Quéré, 1999). Volatile compounds were trapped on a Vocarb 3000 trap (Supelco, Bella Fonte, PA), thermally desorbed at 250 °C and cryofocussed at −100 °C, before being injected and separated on a HP5 capillary column (60 m × 0.32 mm × 1.0 μm film thickness) under the following conditions: carrier gas: helium, 29 $cm\,s^{-1}$ at 35 °C; temperature programme: 35 °C for 5 min, heating rate: 5 $°C\,min^{-1}$ up to 140 °C then 15 $°C\,min^{-1}$ up to 250 °C. Volatiles were detected by a HP7972A quadrupole mass spectrometer (Hewlett Packard) operating in the scan mode within a mass range of m/z 25–300 at 2.5 $scan\,s^{-1}$, after ionisation by electronic impact at 70 eV. They were identified by comparison of mass spectra and retention times with those of standards. When standards were not available, compounds were tentatively identified on the basis of mass spectral data from the Hewlett Packard Chemstation NIST 75K mass

Table 1
Volatile compounds of Emmental cheese juice fermented for 17 days at 24 °C by *P. freudenreichii* and in ripened mini Emmental cheese (P). (C) are control juice and cheese prepared under the same conditions in the absence of *P. freudenreichii*

RT[a] (min)	Compound[b]	QI[c]	In cheese juice[d]			In cheese[d]			Odour threshold value[f]
			C	P	Stat[e]	C	P	Stat[e]	
Alcohols (a.u. or ng g^{-1})[g]		Total number	14	14		8	8		
3.55	Methanol*	TIC	799	1530	**	nd	nd	na	
4.88	Ethanol*	TIC	1996	4001	***	638.3	294.9	NS	
5.23	2-Propanol*	TIC	135.0	112.7	*	358.7	441.4	NS	
6.70	1-Propanol*	TIC	17.6	65.5	***	2.2	3.6	NS	
8.06	2-Butanol*	TIC	40.1	36.7	NS	nd	nd	na	
9.03	2-Methyl-1-propanol*	43	6.3	10.9	***	25.0	10.5	NS	
10.57	1-Butanol*	56	5.0	5.4	NS	0.7	1.3	NS	
11.36	1-Penten-3-ol	TIC	13.2	9.3	*	nd	nd	na	
12.03	2-Pentanol*	TIC	16.9	20.7	NS	nd	nd	na	
13.43	3-Methyl-3-butenol*	68	0.9	1.6	***	nd	nd	na	
13.56	3-Methyl-1-butanol *$	TIC	46.8	355.4	**	59.6	94.2	*	300–4750[w] 3200[m]
13.86	2-Methyl-1-butanol *$	41	13.4	869.1	***	9.2	272.8	***	5500[w] 6250[m]
14.61	4-Methyl-4-penten-2-ol[h]	TIC	7.4	82.2	***	nd	nd	na	
14.96	1-Pentanol*	TIC	10.4	16.3	***	1.4	tr	na	
Aldehydes (a.u. or ng g^{-1})		Total number	9	5		8	8		
6.45	2-Methylpropanal*	TIC	60.7	20.5	**	5.2	2.9	NS	
7.59	Butanal*	TIC	5.4	nd	na	nd	nd	na	
9.92	2-Butenal	TIC	2.0	nd	na	1.2	2.0	NS	
10.09	3-Methylbutanal*$	TIC	61.1	28.8	**	15.9	10. 3	NS	60[w] 60–300[m]
10.50	2-Methylbutanal*$	57	16.6	13.7	NS	9.1	54.5	**	40–130[w] 130[m]
11.89	Pentanal	TIC	4.4	nd	na	1.7	1.2	NS	
20.50	Heptanal*	44	tr	tr	NS	tr	tr	na	
23.07	Benzaldehyde*	TIC	59.8	nd	na	3.4	2.0	*	
24.34	Octanal*	56	tr	tr	NS	tr	tr	na	
Esters (a.u. or ng g^{-1})		Total number	15	26		8	12		
5.66	Methyl acetate*	TIC	99.2	33.7	***	nd	nd	na	
8.48	Ethyl acetate*$	TIC	81.4	148.5	***	9.4	16.5	NS	5000–6000[w] 4700[m]
9.15	Methyl propanoate$	TIC	0.5	250.1	***	<0.9	39.2	na	unk
12.59	Ethyl propanoate*$	TIC	1.7	291.4	***	0.3	271.7	**	4.9–9.9[w]
12.67	n-Propyl acetate*	TIC	1.5	2.5	**	nd	nd	na	
13.03	Methyl butanoate*	TIC	20.4	12.0	***	tr	tr	na	
14.41	Isopropyl propanoate$	75	<0.6	2.6	na	<2.5	15.2	na	unk
15.42	Methyl 2-methylbutanoate	TIC	nd	1.2	na	nd	nd	na	
16.37	Ethyl butanoate*$	TIC	22.7	22.0	NS	12.4	15.8	NS	0.13–450[w] 16[m]
16.73	Propyl propanoate*$	TIC	0.3	5.3	***	<0.6	1.2	*	unk
16.93	Butyl acetate	TIC	2.2	1.3	*	0.7	1.4	**	
18.41	1-Methylpropyl propanoate	57	nd	1.5	na	nd	nd	na	
19.11	Isobutyl propanoate	TIC	nd	24.1	na	nd	nd	na	
19.46	3-Methylbutyl acetate*$	TIC	1.0	4.0	***	14.8	13.3	NS	2[w]
19.56	2-Methylbutyl acetate$	TIC	<0.2	41.6	na	3.9	7.8	***	unk
20.67	Butyl propanoate *	TIC	nd	15.7	na	nd	nd	na	
21.32	Methyl hexanoate	TIC	94.0	29.6	**	nd	nd	na	
23.00	3-Methylbutyl propanoate*$	TIC	<0.2	31.7	na	<2.0	<2.0	na	unk
23.14	2-Methylbutyl propanoate$	TIC	<0.2	404.8	na	<2.0	10.9[i]	na	unk
24.05	Ethyl hexanoate*$	TIC	94.8	73.4	NS	6.2	24.2	NS	1[w]
24.31	Pentyl propanoate	57	0.1	3.6	***	nd	nd	na	
24.96	Methyl heptanoate	TIC	0.9	8.4	***	nd	nd	na	
26.06	3-Methylbutyl butanoate*	TIC	nd	0.3	na	nd	nd	na	
26.16	2-Methylbutyl butanoate	TIC	nd	4.9	na	nd	nd	na	
27.29	Ethyl heptanoate	TIC	0.2	6.8	***	nd	nd	na	
27.49	Hexyl propanoate	TIC	nd	1.9	na	nd	nd	na	
Ketones (a.u. or ng g^{-1})		Total number	16	19		9	12		
4.60	2-Propanone*	TIC	1190	2154	***	304.3	181.7	NS	
7.49	2,3-Butanedione*	TIC	103.6	115.0	NS	15.0	10.6	NS	
7.79	2-Butanone*	TIC	65.8	77.1	NS	63.6	63.6	NS	
10.32	3-Methyl-2-butanone	TIC	1.1	2.2	NS	6.7	2.8	NS	

Table 1 (*continued*)

RT[a] (min)	Compound[b]	QI[c]	In cheese juice[d]			In cheese[d]			Odour threshold value[f]
			C	P	Stat[e]	C	P	Stat[e]	
11.51	2-Pentanone*	TIC	90.0	276.4	***	36.9	40.5	NS	
11.80	2,3-Pentanedione	TIC	13.1	38.8	***	1.3	1.7	NS	
13.84	4-Methyl-2-pentanone*$	43	1.7	13.3	***	3.1	23. 2	***	47[ai]
14.42	3-Methyl-2-pentanone*$	72	<0.5	1.2	na	<2.6	21.0	na	unk
15.93	2-Hexanone*$	TIC	0.9	1.7	*	2.1	28.0	**	4700[ai]
16.08	Cyclopentanone	55, 84	0.2	0.4	**	nd	nd	na	
18.20	2-Methyl-cyclopentanone	TIC	2.6	5.5	***	nd	nd	na	
18.46	3-Methyl-cyclopentanone	69	2.3	5.9	***	nd	nd	na	
19.36	4-Heptanone*	TIC	nd	1.3	na	nd	nd	na	
20.05	2-Heptanone*$	TIC	5.3	1.8	***	90.3	111.6	NS	0.9–3000[w] 700[m] 1300[ai]
20.44	Cyclohexanone	TIC	2.4	1.7	*	nd	nd	na	
22.53	6-Methyl-2-heptanone$	TIC	0.5	0.7	NS	<0.6	2.3	na	unk
22.65	3-Methyl-cyclohexanone	TIC	3.2	8.2	**	nd	nd	na	
22.92	5-Methyl-2-heptanone$	TIC	<0.1	3.0	na	<0.6	25.9	na	unk
23.75	6-Methyl-5-hepten-2-one	TIC	0.8	1.8	*	nd	nd	na	
S-containing compound (a.u.)									
14.13	Dimethyl disulfide*	TIC	7.3	1.1	***	1.8	2.5	NS	
Short-chain acids ($\mu g\,g^{-1}$)		Total number	2	2		5	5		
Acetic acid			500	5620	***	325.3	2407	**	22–100[w]
Propanoic acid			100	14300	***	39.3	6582	***	40.3[w]
Butanoic acid			nd	nd	na	54.0	78.7	*	0.2–6.2[w] 25[m]
Isovaleric acid[j]			nd	nd	na	6.7	45.3	***	0.07–3.2[w]
Hexanoic acid			nd	nd	na	12.0	32.3	*	1–27[w] 14[m]

[a] Retention time in GC-MS.

[b] Compounds marked with * were identified by comparison of RT and mass spectral data with those of authentic compounds, the others by comparison of mass spectral data with those of NIST 75K database.

[c] Quantification ions. TIC, total ion chromatograph peak areas were used or selected fragment(s) as indicated.

[d] Results are means of triplicate (cheese juice) or duplicate (cheese) experiments; nd, not detected; tr, traces.

[e] Results of the analysis of variance: Probability of *F* test: *** $P < 0.001$; ** $0.001 < P < 0.01$; *$0.01 < P < 0.05$; NS, not significant difference; na, ANOVA was not applied.

[f] Flavour threshold values of compounds accurately quantified, given in: [w], water; [m], milk; [ai], air, expressed in $ng\,g^{-1}$, except for acids, in $\mu g\,g^{-1}$; from Sheldon, Lindsay, Libbey, and Morgan (1971); Molimard and Spinnler (1996); Sablé and Cottenceau (1999); unk, unknown.

[g] Values for compounds marked with $ are concentrations, expressed in $ng\,g^{-1}$, or peak areas, expressed in arbitrary units (a.u.) for unmarked compounds. Volatiles were quantified by addition of standard compounds as described in Section 2. Some of them were quantified from the regression curve of a closely related standard, as follows: 2-methyl-1-butanol from 3-methyl-1-butanol; 2-methylbutanal from 3-methylbutanal; methyl propanoate from ethyl acetate; isopropyl propanoate from ethyl butanoate; 3-methylbutyl acetate, 2-methylbutyl acetate, 3-methylbutyl propanoate, 2-methylbutyl propanoate from ethyl hexanoate; 4-methyl-2-pentanone, 3-methyl-2-pentanone, 6-methyl-2-heptanone, 5-methyl-2-heptanone from 2-heptanone; propyl propanoate in cheese from ethyl butanoate; 2-hexanone in cheese from 2-heptanone.

[h] Tentative identification.

[i] 2-Methylbutyl propanoate or 3-methylbutyl propanoate.

[j] Corresponds to the sum of 2-methylbutanoic and 3-methylbutanoic acids, co-eluted under the chromatographic conditions used.

spectral Database. Peaks were quantified by the areas of the total ion chromatogram (TIC) or of selected fragments (m/z) in the case of some co-eluted compounds.

Nine neutral volatile compounds were quantified using calibration curves that were based on the addition of standards to juice or to cheese homogenisate samples, in order to avoid the approximations related to the commonly used internal standard calibration (Drozd, Vodáková, & Koupil, 1990). High purity chemicals (generally higher than 99%) were purchased from Sigma–Aldrich (St. Quentin Fallavier, France): 3-methylbutanal, 3-methyl-1-butanol, ethyl acetate, ethyl propanoate, ethyl butanoate, ethyl hexanoate, propyl propanoate, 2-hexanone, 2-heptanone. Stock solutions of standard compounds or mixtures of them were prepared in high purity (99.8%) methanol at a concentration of 4–10 $mg\,g^{-1}$ each, and stored at −20 °C. An aqueous calibration solution was freshly prepared by diluting stock solutions in boiled deionised water, and was used to spike control juice and cheese homogenisate at eight concentrations ranging from 20 to 2000 $ng\,g^{-1}$ (in juice) or from 0.35–1.4 to 50–200 $ng\,g^{-1}$ cheese (in cheese homogenisate). The concentrations of compounds were calculated from the linear regression curves of the peak areas and added concentrations. Twelve compounds were also quantified from the regression curve of a closely related standard (same chemical function, close molecular mass).

2.4. Analyses of organic acids and amino acids

Lactic acid, acetic and propionic acids of juice were determined by HPLC on an Aminex A-6 ion-exchange column (Bio-Rad, Hercules, CA) at 55 °C with 0.01 N H_2SO_4 as eluent at a flow rate of 1.0 ml min^{-1}. Both UV (210 nm) and refractometric detectors were used. Volatile acids of juice were also analysed with a Varian gas chromatograph (GC, model 3800, Varian, Sugar Land, USA) equipped with a flame ionisation detector and a capillary column (25 m by 0.53 mm; film thickness, 0.5 μm) coated with modified polyethylene glycol (BP21; SGE, Ringwood, Vic., Australia) as previously described (Thierry et al., 2002). Quantification was done from regression curves obtained with standard compounds.

Volatile carboxylic acids of cheeses were extracted by a 50:50 mixture (v/v) of diethyl ether and petroleum ether at acidic pH and analysed by gas chromatography as previously described (Berdagué, Jeunet, & Grappin, 1987). Free fatty acids were extracted from cheese by ether/heptane (50:50, v/v) at acidic pH, separated from fat on an aminopropyl column and analysed by gas chromatography according to De Jong and Badings (1990). These methods did not separate 2-methylbutyric acid from 3-methylbutyric acid, which are referred to as isovaleric acid in the present study. Amino acids of cheese were analysed using an amino acid analyser (AlphaPlus serie 2, Pharmacia, Uppsala, Sweden).

2.5. Statistical analyses

Data for the concentrations of compounds of triplicate (cheese juice) or duplicate (cheese) samples were used for statistical analysis. One-way analyses of variance (ANOVA) were performed by using the General Linear Model procedure of Statgraphics Plus (Statistical Graphic Corp., Englewood Cliffs, NJ, USA) to determine the effect of PAB on the concentration of each compound. Differences between the treatment means were compared at the 5% level of significance using the LSD (least significance difference) test.

3. Results

3.1. Production of volatiles in Emmental cheese juice

Inoculated in cheese juice at 0.6×10^7 cfu (colony-forming unit) ml^{-1}, *P. freudenreichii* TL34 reached 4.5×10^9 cfu ml^{-1} after 17 days. Within the incubation period, the pH increased from 5.4 to 5.7, and PAB consumed 90% of lactic acid (initial concentration 30 mg g^{-1}), with a concomitant production of acetic acid (5.6 mg g^{-1}) and propionic (propanoic) acid (14.3 mg g^{-1}). In addition to the formation of these two acids, PAB significantly modified the profile of neutral volatile compounds of juice.

Sixty-nine volatile compounds were identified in cheese juices by head space GC-MS analyses. The concentrations of 57 of them significantly differed in control juice and in assay juice fermented by PAB (Table 1). The class of compounds showing the highest changes was the esters: eleven esters were detected only in the presence of PAB, and nine other esters were present at higher concentrations. These were essentially esters of propionate (11) and acetate (4), and included branched-chain esters of 2-methyl-1-butanol and, to a lesser extend, of 3-methyl-1-butanol. In contrast, four esters, including three methyl esters were found at a lower concentration in assay juice, whereas the amounts of ethyl butanoate and ethyl hexanoate did not significantly differ in control and assay juices. Nine of the 14 alcohols identified were present at a significantly higher concentration in assay juice: in particular, 2-methyl-1-butanol, 4-methyl-4-penten-2-ol and 3-methyl-1-butanol showed the greatest differences, with amounts in assay juice respectively 65, 11, and 7 times as great as in control juice (Table 1). The concentrations of ketones also significantly differed in control and assay juices, but the changes that were observed were generally far less extensive than those of esters and alcohols. Thirteen ketones out of 19, including four branched-chain ketones and four cycloketones, were present at a higher concentration in assay juice, with 4-methyl-2-pentanone showing the greatest difference. In contrast, most aldehydes (six of nine) and dimethyl disulfide were found at a lower concentration in assay juice. Numerous volatiles produced by PAB (45%) were branched-chain compounds, of various chemical function: alcohols (3-methyl-1-butanol and 2-methyl-1-butanol), acids (isovaleric acid, i.e. 2-methylbutanoic and 3-methylbutanoic acids), aldehyde (2-methylbutanal), esters (mainly esters of branched-chain alcohols) and ketones.

3.2. Production of volatiles in mini Emmental cheeses

P. freudenreichii ITGP22 reached 6×10^9 cfu g^{-1} in the assay cheeses, whereas indigenous PAB remained below 5×10^6 cfu g^{-1} at the end of ripening in control cheese.

The gross composition of the cheeses ($63.9 \pm 0.6\%$ total solids, $46.2 \pm 0.4\%$ fat in dry matter and $51.4 \pm 1.2\%$ moisture in non-fat cheese) was consistent with the expected values for this type of mini-cheese (Buisson et al., 1987; Richoux et al., 1998). Neither cheese composition nor proteolysis indices (pH 4.6-soluble N, 12% TCA-soluble N and free amino acids) were significantly affected by the presence of PAB (results not shown). In contrast, the amino acid profile was influenced by the presence of PAB. In the assay cheese, asparagine was not detectable at the end of ripening and

the concentration of aspartic acid was only 28 ± 6.8 mg kg^{-1}, versus 598 ± 25 mg g^{-1} (Asn) and 184 ± 6 mg kg^{-1} (Asp) in control cheese. In contrast, the concentrations of tyrosine, proline, leucine and isoleucine were significantly ($P < 0.05$) higher is the assay cheese than in control cheese (respectively 16%, 15%, 11% and 10% more). Lipolysis was markedly enhanced by PAB: assay cheeses contained 5.6 times more free fatty acids than control cheeses. The longest fatty acids (C14:0–C18:3) were more affected than the shortest ones (C4:0–C12:0) (results not shown).

Forty-one volatiles were identified in cheese by head space GC-MS. All of them were also found in cheese juice. The effect of PAB on the profile of volatile compounds of cheese was less pronounced in cheese than in juice. However, 15 compounds were found at a greater concentration, and one at a lower concentration (benzaldehyde) in the presence of PAB. Most generally, the trends of variations were similar to those observed in juice: seven esters (including five esters of propionate), two branched-chain alcohols (2-methyl- and 3-methyl-1-butanol), one branched-chain aldehyde (2-methylbutanal) and five ketones (including four branched-chain ketones: 3-methyl- and 4-methyl-2-pentanone, 5-methyl- and 6-methyl-2-heptanone) were present at a higher concentration in assay cheese (Table 1). In contrast to what was observed in juice, the concentrations of most straight-chain ketones, all alcohols except 2-methyl-1-butanol and 3-methyl-1-butanol and aldehydes, except 2-methylbutanal, were not significantly affected by the presence of PAB.

3.3. Quantification of selected volatile compounds

The nine standard compounds chosen (see Section 2) presented good linear regressions when added either to juice or to cheese homogenisate, with values of r (coefficient of correlation) most generally higher than 0.99. The slopes of regression curves varied between 0.96 and 2.69 in cheese juice, and from 0.13 to 0.65 in cheese homogenisate. The slopes calculated from spiked juices were twice (3-methylbutanal) to 13 times (ethyl hexanoate) higher than those obtained from spiked homogenisates. This means that the volatile compounds were more easily extracted from juice than from homogenisate by headspace extraction, very likely because cheese homogenisate contained fat and consequently retained better hydrophobic compounds than a purely aqueous sample, such as cheese juice.

Regression curves were used to quantify 21 compounds, selected among those showing the largest differences in concentration in control and assay cheeses, and among known flavour-active compounds of Emmental cheese. Comparing, for each compound, its concentration in cheese with its odour threshold value, gives a first idea of the possible contribution of this compound to cheese flavour. Hence, among the quantified compounds for which an odour threshold value was available, the most probable flavour-active compounds were short-chain carboxylic acids, 2-methylbutanal and esters. PAB largely contributed in particular to the formation of acetic, propionic and isovaleric acids, 2-methylbutanal, and esters of propionic and acetic acids, whereas the amounts of more common esters of cheese such as ethyl butanoate and ethyl hexanoate were not significantly affected by their presence.

3.4. Comparison of juice and cheese volatile profiles

Forty-one volatiles were common in juice and cheese. The profile of juice volatiles additionally contained in 28 compounds that were not detected in cheese (Table 1). This could result either from the formation of original compounds in juice but not cheese, or from variations in the ability of PAB strains to form volatile compounds, or from an easier extraction of volatiles from juice than from cheese homogenisate, or a combination of these factors. However, the latter hypothesis appears very probable since calibration curves showed that all the standard compounds spiked in cheese juice and cheese homogenisate were more easily extracted from juice than from cheese homogenisate (containing fat). The largest differences in extraction were observed for the most hydrophobic compounds. Half of the compounds that were specifically found in juice were esters, including seven long-chain esters (molecular mass >144, retention time >23 min under our experimental conditions) (Table 1). As regards the four methyl esters found in juice but not in cheese, their presence in juice can be related to the high amount of the corresponding alcohol (methanol) in juice and not in cheese (Table 1).

4. Discussion

PAB induced the increase or the appearance of numerous volatile compounds in juice and in cheese. Most of them had already be found either in Swiss-type cheeses (Bosset, Gauch, Mariaca, & Klein, 1995; Maarse, Visscher, Willemsens, Nijssen, & Boelens, 1994), or, for a few of them, in other cheese varieties: methyl propanoate and 2-butenal in Cheddar cheese, 1-penten-3-ol, methyl heptanoate, cyclohexanone in Parmesan cheese (Maarse et al., 1994), and 4-heptanone in goat cheese (Maarse et al., 1994). Some compounds produced in juice by PAB had not previously been detected in cheese: methyl-cyclopentanones and methyl-cyclohexanones are generally present in meat (Maarse et al., 1994), whereas 4-methyl-4-penten-2-ol has not previously been found in food products, to our knowledge.

The changes that PAB induce in the volatile profile show large similarities in juice and in cheese. Fewer significant effects were, however, observed in cheese, probably because the activities of lactic starters interfered with those of PAB. The use of a pure culture of PAB demonstrates their ability to form numerous volatile compounds, in the absence of interfering microflora.

The changes in volatile profile induced by PAB mainly concern three groups of volatiles: short branched-chain compounds (19 compounds), esters (13 esters), and carboxylic acids. Short branched-chain compounds are thought to derive from the corresponding branched-chain amino acids, leucine, isoleucine and valine (Yvon & Rijnen, 2001). PAB are able to convert these amino acids to the corresponding carboxylic acids and alcohols (Thierry et al., 2002). They can also reduce branched-chain aldehydes to the corresponding alcohols (Thierry & Maillard, 2002). In juice and cheese samples with PAB, the products originating from isoleucine catabolism (2-methyl-1-butanol, 2-methylbutanal, 2-methylbutyl propanoate) were found at higher concentrations than the corresponding products of leucine and valine, whereas the opposite was true in control samples. This effect of *P. freudenreichii* on the formation of isoleucine-derived products has already been pointed out (Thierry & Maillard, 2002). As regards the branched-chain ketones produced in the presence of *P. freudenreichii,* all five have already been found in Swiss-type cheeses (Maarse et al., 1994), but their origin, to our knowledge, is unknown.

The second group of compounds largely affected by PAB were esters. All but three of these esters have already been found in Swiss-type cheeses (Bosset et al., 1995; Maarse et al., 1994). The three remaining esters were branched-chain esters (isopropyl propanoate, methyl 2-methylbutanoate, and 2-methylbutyl acetate) and are commonly found in fruits (Maarse et al., 1994). Esters are particularly abundant in hard cheeses such as Comté and Parmigiano Reggiano (Bosset & Gauch, 1993; Maarse et al., 1994). Their synthesis is generally attributed to the activity of cheese microflora (Cristiani & Monnet, 2001), but purely chemical mechanisms of synthesis have also been hypothesised, at least in cheeses which are ripened for a long period of time (Adda, Gripon, & Vassal, 1982). Whatever their mechanism of formation, micro-organisms are essential for producing the carboxylic acids (or the acyl coA) and the alcohols that are required for ester synthesis. The results we observe in juice suggest that PAB were very likely involved in ester synthesis, i.e. did not only act as producers of precursors, since no synthesis of esters occurred during the incubation of control juice, although some ester precursors (carboxylic acids and alcohols) were present in juice before incubation (results not shown). However, PAB essentially affected the formation of the esters of acetic and propionic acids, which are their main fermentation products, whereas they did not influence the amounts of the esters of other acids, such as butanoic and hexanoic acids.

The third group of compounds were carboxylic acids, which have varied origins. They are formed from lactate fermentation (acetic and propionic acids), from lipolysis (butyric, hexanoic, and higher-chain fatty acids), and from amino acid catabolism (isovaleric acid). Our results are in accordance with recent works showing the prominent role of propionibacteria in the formation of free fatty acids and isovaleric acid in Emmental cheese (Chamba & Perreard, 2002; Hettinga & Reinbold, 1972; Thierry et al., 2003). In the present study, the influence of PAB on lipolysis was more pronounced than that observed by Chamba and Perreard (2002) in full size Emmental cheese (respectively, $\sim$5 times and $\sim$2 times more free fatty acids in assay cheese than in control cheese).

Several compounds produced by PAB could be involved in cheese flavour. Some of them have already been identified as flavour compounds in Emmental cheese: acetic, propionic, and butanoic acids, and 2-methyl and 3-methylbutanal (Preininger & Grosch, 1994; Preininger, Warmke, & Grosch, 1996; Rychlik, Warmke, & Grosch, 1997; Warmke, Belitz, & Grosch, 1996). The amounts of volatile acids found in mini cheeses were similar to the data available for Emmental cheese, except for isovaleric acid, found at a concentration 3–4 times greater than in Emmental cheese (Preininger et al., 1996; Rychlik et al., 1997). In contrast, the concentrations of neutral flavour compounds in mini cheese were 2–10 times lower than those of Swiss Emmental cheese, which can be explained by a longer ripening time of the latter cheese. Unfortunately, the most potent odorants found by the above authors, i.e. methional, 4-hydroxy-2,5-dimethyl-3(2H)-furanone and 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, were either not extracted or not present in our samples, which prevents us from drawing a conclusion about a possible involvement of PAB in their formation. Esters are also known to play an important role in flavour, and sometimes off-flavour, of cheese. It is interesting to note that ethyl butanoate and ethyl hexanoate, two common esters of cheese that are also considered as flavour compounds of Emmental cheese (Preininger & Grosch, 1994, 1996), were not significantly influenced by the presence of PAB, in juice cultures on in cheeses. However, some other esters formed in the presence of PAB could also be involved in cheese flavour. Ethyl propanoate, for example, was found at a 10 times higher concentration in cheese than ethyl butanoate, and has a low odour threshold value. In addition, branched-chain ketones may also contribute to cheese flavour. Their odour threshold values in water or milk are not available, but 4-methyl-2-pentanone has an odour threshold value in

air 100 times lower than that of the corresponding straight-chain ketone.

In conclusion, *P. freudenreichii* produces flavour compounds of varied origins (fermentation, lipolysis, amino acid catabolism) in Emmental cheese. The mechanisms and the strain-dependence of the formation of these compounds has now to be investigated.

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