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ORIGINAL PAPER

The role of propionibacteria in the volatile profile of Pategrás cheeses

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Abstract Pategrás is a typical semi-hard cheese of Argentina. Over the years, successive technological changes have been applied to its manufacture, being the addition of propionibacteria (PAB) one of the most relevant. The aims of the present work were to determine some physicochemical parameters and volatile composition of commercial samples and evaluate the effect of PAB on volatile profile during ripening, by comparing cheeses made at pilot scale, with or without added PAB. Commercial samples displayed some differences in terms of rough composition, mainly ripening degree. Acids, especially short-chain fatty acids (C_2 to C_4), were the most representative group of volatile compounds, being propanoic acid the most abundant. Alcohols and ketones were other chemical groups that prevailed in the volatile profiles whereas esters and aldehydes constituted a minority fraction. For cheeses made at pilot scale, the levels of several compounds such as diacetyl, acetoin, primary alcohols, ethyl esters, branched-chain aldehydes, branched-chain alcohols, branched-chain acids, and short-chain fatty acids were significantly influenced by the presence of PAB. Most of the changes observed in the volatile fraction during ripening and the differences found between cheeses made with and without added PAB were in agreement with published data. This work provides detailed information about the volatile composition of Pategrás cheeses made with the inclusion of PAB, which contribute to the characterization of the most traditional Argentinean semi-hard cheese.

Keywords Volatile profile · Physicochemical composition · Propionibacteria · Pategrás cheese

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1 Introduction

Pategrás is the most typical semi-hard cheese of Argentina made from pasteurized bovine milk. According to Argentinean Legislation, the main sensory characteristics of aged Pategrás cheeses are the semi-hard and elastic consistency with small holes or eyes well distributed throughout the matrix and the characteristic and slightly salty flavor. The ripening period is dependent on the cheese block size, from 30 to 60 days (ANMAT 2014). In the past, Pategrás cheese was manufactured using natural milk starters prepared by warming good-quality raw milk at about 65 °C followed by an immediate cooling to 45 °C and leaving at this temperature until milk pH decreased to a value of 5.2. Then, this natural milk starter was cooled to 8–9 °C and used within 24 h. This procedure allowed to obtain a starter composed mainly by natural thermophilic streptococci. Today, to improve and standardize the quality of the natural milk starter, they have been replaced by DVS cultures (direct-vat-set) of mesophilic or thermophilic bacteria. However, the pasteurization of milk and the use of commercial cultures have been conducted to reduce the eye development by heterofermentative bacteria provided by raw milk or natural starter. The concomitant loss of the typical sensory characteristics of this cheese was considered as a negative effect for consumers. Thus, in order to confer unique flavor attributes to Pategrás cheese, this traditional procedure of cheese making was replaced by the more recent that includes the use of *Propionibacterium freudenreichii* strains as adjunct cultures (Zalazar et al. 1999; Perotti et al. 2009). In our country, the technology of manufacture of "cheeses with eyes" still represents a major challenge to dairy industries, which has led to the development of protocols for cheese making (Gauna 2005).

Propionibacteria (PAB) and in particular *P. freudenreichii* constitutes one of the major microflora present in Swiss-type cheeses, a variety of cheeses with characteristic round "eyes," such as Emmental, Comté, Gruyère, Appenzeller, and Massdam. In these cheeses, PAB are often added to milk as adjunct cultures, but sometimes they are indigenous to the raw milk (Beuvier et al. 1997; Thierry et al. 2005a). *Propionibacterium* species can also be used in the manufacture of some cheeses without eyes to enhance flavor formation (Fernandez-Espla and Fox 1998; Thierry et al. 2005a). In addition to the ability to ferment lactic acid to acetic and propanoic acids, more recently, PAB have been demonstrated to play a key role in the formation of free fatty acids and several branched-chain volatile compounds (Thierry et al. 2011). However, these properties are highly strain dependent. In fact, data from literature revealed that the degree in which PAB can influence the sensory characteristics of cheeses is highly dependent on cheese composition (mainly NaCl content) and ripening temperature. For example, PAB had a lesser effect on the volatile profile of Morbier cheese than that of Swiss cheese (Thierry and Maillard 2002).

The studies carried out on Pategrás cheeses by our research group have been focussed on their suitability as food matrix for the incorporation of probiotic bacteria (Bergamini et al. 2005, 2010a; Perotti et al. 2009). However, the effect of the addition of *P. freundenreichii* during cheese making on ripening parameters has not yet been investigated. In particular, no information is available concerning the volatile profile of Pategrás cheese. So, the objectives of the present work were to do a preliminary study about physicochemical parameters and the volatile compounds that characterize this

cheese variety by analyzing commercial samples, and to evaluate the effect of PAB on volatile profile during ripening in cheeses made at pilot scale.

2 Materials and methods

2.1 Commercial cheese samples

A total of 10 samples of Pategrás cheeses (approximately 500 g from wheels of 1 to 5 kg) from leading commercial brands were purchased in the local markets of Santa Fe City. According to data from manufacturers, cheeses were made with added PAB.

2.2 Cheese-making protocol

Two replicate cheese-making trials were manufactured on two consecutive days (using the same milk) at the pilot plant of Instituto de Lactología Industrial (INLAIN) located in Santa Fe (Argentina) according to a standard protocol described by Zalazar et al. (1999). Two types of cheeses were obtained in each day; reference (*R*) cheeses manufactured with the older technology of Pategrás cheeses, i.e., without PAB, and experimental (*E*) cheeses made with added PAB. The presence of PAB in cheeses *E* required other ripening conditions to allow the growth of these microorganisms.

Raw bulk cow's milk (fat 3.4 ± 0.2%, protein 3.1 ± 0.1%, pH 6.7 ± 0.05, acidity 14 ± 1°D) was supplied by a nearby dairy plant (Milkaut SA, Santa Fe, Argentina). In each cheese-making day, milk (150 L) was pasteurized at 65 °C for 20 min and cooled to 35 °C, and then, $CaCl_2$ was added to give a final concentration of 0.2 $g.L^{-1}$. After that, milk was divided into two equal parts. A mixed culture composed by *Streptococcus thermophilus* (ST-M5) and *Lactobacillus helveticus* (LH-B02) (85:15) (Chr. Hansen, Argentina) was used as a starter in cheeses *R*, which were added in a dose to achieve a concentration of 10^7 $CFU.mL^{-1}$. In the case of cheeses *E*, the starter was composed of a mix of *S. thermophilus* (ST-M5), *L. helveticus* (LH-B02) and *P. freudenreichii* PS-1 (60:10:30) (Chr. Hansen, Argentina). After 15 min, chymosin (Chymax, Chr. Hansen, Argentina) (0.35 $mL.L^{-1}$ of milk) was added to each vat in order to allow the coagulation process in 10 min. Once the appropriate strength is reached, the coagulum was cut in several steps (with intermittent manual stirring) until the curd particles reached a corn grain size (8–10 mm). Then, a washing step of curd was performed: 15 L of supernatant whey were replaced by water at 35 °C. After 10 min, the curd grains and the whey were gently stirred and heated to 49 °C at 0.5 $°C.min^{-1}$. Curd grains were maintained at this temperature for 10–15 min approximately, to promote syneresis. Finally, the curd was placed into the molds, which were pressed (0.2–0.3 $kg.cm^{-2}$) for 18 h. Cheeses of approximately 6–7 kg were brined (20% *w*/*v*, pH 5.4) at 12 °C for 24 h. Cheeses *R* were ripened at 12 °C and 80% relative humidity for 60 days while cheeses *E* were ripened in three steps: at 12 °C (cold room) for 15 days, then at 24 °C (warm room) for approximately 15 days, and finally at 12 °C for 30 days.

2.3 Cheese sampling

Samples from market were analyzed at commercialization time; therefore, the ripening time is unknown. Argentinean Legislation states that cheeses from wheels of 1 to 5 kg must be ripened at least for a minimum time of 45 days. Samples were cut into cubes and grated using a food processor.

Cheeses made at pilot scale were sampled for volatile compound analysis at three times (35, 45, and 60 days) according to the ISO (2008b). The sampling times were chosen after cheeses *E* had left the warm room, since from this moment the ripening temperature was the same for both types of cheeses (12 °C). Gross composition and pH were determined only at the end of ripening (60 days). On each sampling time, two cheeses from each type were sampled.

2.4 Compositional analysis

Samples were analyzed in duplicate for moisture (oven drying at 102 °C; ISO 2004), protein (TN × 6.38) (Kjeldhal method; ISO 2011), fat (Gerber-Van Gulik butyrometric method; ISO 2008a, b), pH (potentiometric method; Bradley et al. 1993), and sodium (atomic absorption spectrometric method; ISO 2007). Soluble nitrogen (SN) at pH 4.6 was analyzed according to Gripon et al. (1975). Ripening degree was calculated as the ratio SN pH 4.6/TN*100.

2.5 Volatile compound analysis by SPME-GC-FID/MS

Volatile compounds were isolated by headspace-solid phase microextraction (HS-SPME) and analyzed by gas chromatography (GC) coupled to flame ionisation detector (FID) and mass spectrometry (MS) detector.

Cheese samples (5 g) were placed into 30-mL glass vials, which were hermetically sealed with aluminum seal and butylteflon septa. Vials were maintained at 40 ± 1 °C for 10 min, and then a 1 cm × 50/30 μm Stable-Flex DVB/CAR/PDMS (Supelco, Bellefonte, PA, USA) fiber was exposed for 15 min into the headspace. After the extraction step, the analytes were thermically desorbed from the fiber into the injector port at 250 °C for 5 min (splitless mode). A Perkin Elmer gas chromatograph model 9000 equipped with a FID was employed for the separation of analytes. Chromatographic separation was performed on a HP-Innowax fused silica capillary column (60 m length × 0.25 mm i.d., 0.25 μm film thickness; Agilent J&W, CA, USA) under the following conditions: temperature program 45 °C (4 min), then 8 °C.min^{-1} to 150 °C (3 min), and finally 10 °C.min^{-1} to 250 °C (5 min); detector temperature 290 °C; hydrogen was used as a carrier gas at a constant flow rate of 2 mL.min^{-1}.

Samples were also analyzed by MS using a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 2000 ion trap mass detector (Varian Inc., Palo Alto, CA, USA). The eluted compounds were separated on a Vf-5ht column (Agilent, CA, USA) (30 m × 0.25 mm × 0.10 μm). The chromatographic conditions employed were the same as those described for GC-FID analysis. MS was operated in the electron impact (EI) mode with 70 eV of energy, transfer line temperature of 250 °C, mass range from 40 to 350 amu, and scan rate of 0.5 scan.s^{-1}. Helium was used as a carrier gas at a flow rate of 1 mL.min^{-1}. Peak recognition was performed by comparison with mass spectra

from both the library database provided by software (NIST 98, Gaithersburg, MD, USA; Wiley libraries, Hoboken, NJ, USA) and the standard compounds (Sigma-Aldrich, Milan, Italy) analyzed under the same analytical conditions.

The comparison of data from GC-MS and GC-FID revealed that, in the assay conditions, both a higher number and a better resolution of peaks were recorded by GC-FID than GC-MS. For this reason, authentic standards of compounds identified by GC-MS and others commonly reported in cheeses were injected in the GC-FID system and their retention times were obtained. The identification of peaks from cheese samples was conducted by matching the retention times with those of standards. A more reliable identification was performed by calculating linear retention index (LRI) values. For this purpose, a series of *n*-alkanes (Supelco, Bellefonte, PA, USA) was injected under the same chromatographic conditions as for samples and the values were obtained according to the expression proposed by Van den Dool and Kratz (1963). LRI were compared with published data (Mallia et al. 2005; Goodner 2008).

Peak areas of compounds from GC-FID were integrated. Response factors were determined for each component by the external standard method using 3-point curves constructed with solutions of standard compounds prepared at different concentrations. Authentic standards of acetaldehyde, 3-methylbutanal, benzaldehyde, 2-propanone, 2-butanone, 2-pentanone, 2,3-butanedione, 2-hexanone, 2-heptanone, 3-hydroxy-2-butanone, 2-nonanone, 2-undecanone, 2-propanol, ethanol, 1-propanol, 2-pentanol, 1-butanol, 3-methyl-1-butanol, 1-pentanol, 2-heptanol, 1-hexanol, 2-ethyl-1-hexanol, 1-octanol, ethyl acetate, ethyl butanoate, ethyl propanoate, methyl butanoate, 3-methylbutyl acetate, ethyl hexanoate, ethyl octanoate, 2-methylpropanoic acid, propanoic acid, butanoic acid, 3-methylbutanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, decanoic acid, and dodecanoic acid from Sigma-Aldrich Inc. (Milan, Italy) and acetic acid from Merck (Darmstadt, Germany) were accurately weighed and dissolved in appropriate solvent (ethyl ether, hexane or 2-propanol). Stock solutions were prepared (500 to 2000 $\mu g.mL^{-1}$) for each chemical group of compounds (aldehydes, ketones, alcohols, esters, and acids). Two additional calibration standard solutions were prepared by further dilution. When standards were no available (2-methyl butanal, phenylacetaldehyde, 2,3-butanediol, phenylmethanol, nonanoic acid), an estimated response factor was calculated from suitable homologues. The identified compounds were semi-quantified using these factors and the area values; the results were expressed as micrograms per kilogram. It is important to notice that these values are not true concentrations since the efficiency of extraction of each compound by fiber and rate of release of volatiles from cheeses are not considered. However, for comparative studies, these calculated values are more reliable and can be used to establish differences between cheeses.

2.6 Statistical analysis

Gross chemical composition and pH values were compared between cheeses (*R* and *E*) at the end of ripening (60 days) using one-way analysis of variance (ANOVA). Data from volatile profiles were subjected to ANOVA at each time of sampling (35, 45, and 60 days). Statistical analysis was performed using statistical packages for Windows

(version 10.0, SPSS Inc., USA). Differences between the treatment means were compared at 5% level of significance by using LSD (least significance difference) test.

3 Results and discussion

3.1 Compositional analysis

Gross composition (means ± standard deviations) of cheese samples are presented in Table 1. Moisture and fat in dry matter ranged from 36 to 41 g.100 g^{-1} and 45 to 51 g.100 g^{-1}, respectively. These values were consistent with those established by the Argentinean Legislation (ANMAT 2014) for semi-hard cheeses (moisture between 36 and 46%) and fatty cheeses (fat in dry matter between 45 and 60%). pH ranged from 5.40 to 5.80 and the protein content had values comprised between 25 and 32 g.100 g^{-1}. The NaCl content ranged between 1.0 and 1.8 g.100 g^{-1}, which corresponded to values of salt in moisture from 2.7 to 5.0%. Salt level is a key parameter for both the microbiological and physicochemical characteristics of cheeses since it affects a_w, the composition of the microbial population and certain enzymatic activities. In particular, the levels of NaCl had an important effect on the growth of PAB. It has been reported that high salt-in-moisture contents can delay PAB growth and decrease the volatile fatty acid content, methylbutyric acid formation, and CO_2 production (Richoux et al. 1998; Thierry et al. 2004a, b, 2005a, b). However, this inhibiting effect is strain dependent. Ripening degree markedly differed among commercial samples (13 to 32%). The wide range of values in the ripening degree found in Pategrás samples is a fact commonly observed in different Argentinean cheese varieties (Wolf et al. 2010, 2011). The main reason is the lack of standardization in the technological process. Each industry uses different milks, ingredients, primary and secondary starters, and cheese-making procedures, which are sources of variations in

Table 1 Gross chemical composition and pH values of Pategrás cheeses

Parameters	Commercial cheeses		Cheeses analyzed at a pilot scale	
	Mean	Range	*R*	*E*
pH	5.66 ± 0.15	5.40–5.80	5.30 ± 0.07a	5.45 ± 0.10a
Moisture (%)	37.8 ± 1.5	36.0–40.5	41.3 ± 0.7a	38.2 ± 0.9a
Fat (dry matter) (%)	47.3 ± 1.7	44.9–51.0	49.5 ± 1.3a	53.3 ± 2.6a
Protein (dry matter) (%)	26.9 ± 1.9	25.0–31.4	23.9 ± 0.7a	22.4 ± 0.9a
Ripening degree (%)	21.3 ± 5.8	13.0–32.1	16.4 ± 1.3a	18.7 ± 1.7a
NaCl content (%)	1.55 ± 0.23	1.07–1.77	1.53 ± 0.05a	1.55 ± 0.10a

Data reported of commercial cheeses are average values ± standard deviation (SD) of 10 samples analyzed in duplicate at commercialization time

The range corresponds to minimum and maximum values

R and *E* reference and experimental cheeses, respectively. Data reported are average values ± SD of two cheese-making trials analyzed in duplicate at the end of ripening (60 days)

Means in each column with different letters are significantly different ($P < 0.05$)

gross composition and extension of proteolysis during ripening. Besides, ripening times of commercial cheeses can vary markedly. The values of compositional analysis were in accordance with those reported for other semi-hard cheeses (Zalazar et al. 1999; Ramonda 2009). On the other hand, values of pH, fat matter, moisture, protein, salt content, and ripening degree did not show statistical differences ($P > 0.05$) between cheeses *R* and *E*. Thus, the gross composition of Pategrás made at pilot scale was not influenced by the addition of PAB. Similar results were reported by Thierry et al. (2004a, b, 2005a, b) in Emmental and Raclette cheeses.

3.2 Volatile compound profiles

3.2.1 Commercial samples of Pategrás cheeses

Individual volatile components detected in the commercial samples of Pategrás cheeses are listed by chemical category in Table 2. For each compound, mean values ± standard deviations (SD) and range of values (minimum and maximum values) were also included. All these compounds have been already reported in different varieties of cheeses containing PAB (Bosset et al. 1993; Thierry et al. 1999, 2004a, 2005a, b, 2006; Rychlik and Bosset 2001a, b; Mallia et al. 2005). Figure 1 shows the relative percentages of the different chemical groups calculated with respect to the total content of identified compounds.

As can be seen, except for S3 and S5 samples, the volatile fraction of cheeses was very rich in *acids*; the values found ranged from 50 to 72% of the total content of compounds. Among the acid groups, propanoic, acetic, and butanoic acids ranked in order of decreasing levels. In particular, propanoic acid reached values greater than 50% of the acid fraction in the majority of samples. 2-Methylpropanoic and 3-methylbutanoic acids were also identified in all samples, being a minority fraction. The preponderance of acids and the high levels of propanoic acid have been extensively reported in cheeses containing PAB (Engels et al. 1997; Thierry et al. 2004a, 2005a, b). Short-chain fatty acids and some branched-chain fatty acids such as 2- and 3-methylbutanoic are those most likely involved in Swiss-type cheese flavor (Bosset et al. 1993; Thierry et al. 2004b). PAB are known to ferment lactic acid to acetic and propanoic acids, but more recently it was shown that they play a key role in the formation of free fatty acids from lipolysis of the milk fat or branched-chain fatty acids from leucine or isoleucine catabolism (Thierry et al. 2004a).

Alcohols and *ketones* were other important groups from a quantitative viewpoint. Alcohols presented percentual values that ranged from 7 to 55%. For the ketone group, values higher than 10% were found in the majority of samples. In particular, a preponderance of alcohols was observed in the volatile profile of S3 and S5 samples, whereas ketones had a greater incidence in the S8 sample. Alcohols constituted a diversified group of compounds. Among them, primary linear-chain alcohols, secondary linear-chain alcohols, branched-chain alcohols, and one aromatic alcohol were detected (Table 2). In accordance with the finding in other types of cheeses, ethanol was the most abundant alcohol (Engels et al. 1997; Bergamini et al. 2010b). Ethanol is a direct product of lactose fermentation, but it can be also derived from the reduction of aldehydes formed from the amino acid catabolism (McSweeney and Sousa 2000). 3-Methyl-1-butanol, which is presumably originated from leucine, was the second most

Table 2 Volatile compounds identified in commercial samples of Pategrás cheeses

Volatile compounds	LRI	Levels[a] (μg.kg^{-1})	Range[b]	*n*	Method of identification[c]
Aldehydes					
Acetaldehyde	668	0.19 ± 0.25	n.d—0.68	7	ST, LRI
2-Methylbutanal	909	0.06 ± 0.07	n.d—0.19	7	ST, LRI, MS
3-Methylbutanal	914	0.03 ± 0.04	n.d—0.10	5	LRI, MS
Benzaldehyde	1537	0.08 ± 0.11	n.d—0.36	8	ST, LRI, MS
Phenylacetaldehyde	1736	0.02 ± 0.02	n.d—0.05	8	ST, LRI, MS
Ketones					
2-Propanone	811	0.30 ± 0.17	0.08—0.61	10	ST, LRI, MS
2-Butanone	902	0.16 ± 0.15	0.04—0.56	10	ST, LRI, MS
2-Pentanone	968	0.41 ± 0.51	0.03—1.37	10	ST, LRI, MS
2,3-Butanedione	974	0.65 ± 0.62	0.03—2.20	10	ST, LRI, MS
2-Hexanone	1079	0.01 ± 0.01	n.d—0.03	6	ST, LRI
2-Heptanone	1180	0.14 ± 0.06	0.05—0.22	10	ST, LRI, MS
3-Hydroxy-2-butanone	1291	2.48 ± 1.70	0.40—5.05	10	ST, LRI, MS
2-Nonanone	1391	0.01 ± 0.01	n.d—0.03	7	ST, LRI, MS
2-Undecanone	1603	0.001 ± 0.004	n.d—0.01	1	ST, LRI, MS
Alcohols					
2-Propanol	923	0.11 ± 0.14	n.d—0.36	7	ST, LRI, MS
Ethanol	934	6.40 ± 4.92	1.29—15.17	10	ST, LRI
1-Propanol	1041	0.32 ± 0.42	n.d—1.21	9	ST, LRI, MS
2-Pentanol	1121	0.22 ± 0.46	n.d—1.47	4	ST, LRI
1-Butanol	1150	0.14 ± 0.11	0.01—0.31	10	ST, LRI, MS
3-Methyl-1-butanol	1210	0.36 ± 0.17	0.08—0.60	10	ST, LRI
1-Pentanol	1252	0.02 ± 0.01	n.d—0.05	9	ST, LRI
2-Heptanol	1323	0.02 ± 0.04	n.d—0.14	4	ST, LRI, MS
1-Hexanol	1357	0.01 ± 0.01	n.d—0.03	3	ST, LRI
2-Ethyl-1-hexanol	1495	0.01 ± 0.01	n.d—0.04	4	ST, LRI, MS
2,3-Butanediol	1546	0.20 ± 0.31	n.d—0.94	4	LRI
1-Octanol	1567	0.01 ± 0.01	n.d—0.03	2	ST, LRI, MS
Phenylmethanol	1892	0.03 ± 0.03	n.d—0.07	7	LRI, MS
Esters					
Ethyl acetate	882	0.29 ± 0.45	0.02—1.36	10	ST, LRI, MS
Ethyl propanoate	955	0.25 ± 0.25	0.03—0.83	10	ST, LRI, MS
Methyl butanoate	982	0.11 ± 0.08	n.d—0.22	8	ST, LRI
Ethyl butanoate	1034	0.32 ± 0.19	0.09—0.65	10	ST, LRI, MS
3-Methylbutyl acetate	1118	0.01 ± 0.01	n.d—0.03	6	ST, LRI, MS
Ethyl hexanoate	1236	0.07 ± 0.08	n.d—0.24	7	ST, LRI, MS
Ethyl octanoate	1434	0.01 ± 0.02	n.d—0.04	4	ST, LRI, MS
Acids					
Acetic acid	1465	3.39 ± 0.96	2.27—4.73	10	ST, LRI, MS
Propanoic acid	1554	9.36 ± 4.00	2.17—14.97	10	ST, LRI, MS

Table 2 (continued)

Volatile compounds	LRI	Levels[a] (μg.kg^{-1})	Range[b]	*n*	Method of identification[c]
2-Methylpropanoic acid	1583	0.16 ± 0.11	n.d—0.35	9	ST, LRI
Butanoic acid	1640	1.80 ± 1.39	0.57—5.33	10	ST, LRI
3-Methylbutanoic acid	1682	0.23 ± 0.21	0.05—0.65	10	ST, LRI
Pentanoic acid	1747	0.01 ± 0.01	n.d—0.02	7	ST, LRI
Hexanoic acid	1861	0.45 ± 0.12	0.29—0.63	10	ST, LRI
Heptanoic acid	1966	0.02 ± 0.05	n.d—0.15	5	ST, LRI
Octanoic acid	2073	0.12 ± 0.05	0.06—0.21	10	ST, LRI
Nonanoic acid	2183	0.03 ± 0.05	n.d—0.14	4	LRI
Decanoic acid	2286	0.05 ± 0.02	0.02—0.09	10	ST, LRI
Dodecanoic acid	2494	0.02 ± 0.02	n.d—0.07	5	ST, LRI

n number of samples in which volatile compounds were identified

[a] Data reported are average values ± standard deviation (SD) of samples analyzed in duplicate ($n = 10$)

[b] Range of values of volatile compounds (minimum and maximum values)

[c] Identification of compounds was performed on the basis of the following criteria: LRI—comparison of LRI calculated with data published in the literature; MS—comparison of mass spectral data with those of library compounds; and ST—comparison of retention time with those of authentic standards

abundant alcohol in the majority of samples. This compound has a pleasant aroma of fresh cheese (Curioni and Bosset 2002), and it has been detected at substantial levels in cheeses containing PAB (Engels et al. 1997; Thierry and Maillard 2002; Thierry et al. 2004a, 2005a). Methyl ketones (from C_3 to C_{11}), 2,3-butanedione (or diacetyl), and 3-

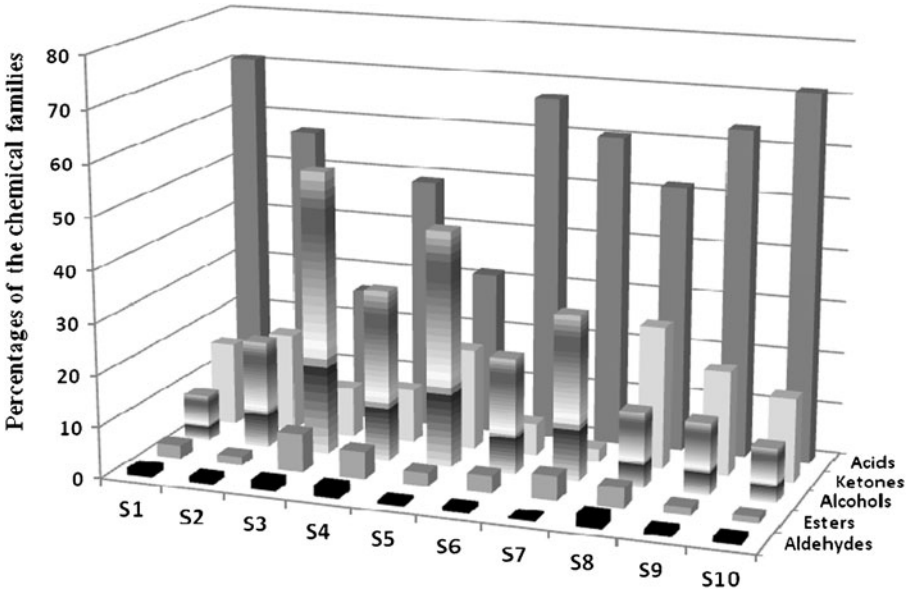

Fig. 1 Relative percentages of the different chemical classes of volatile compounds in commercial samples of Pategrás cheeses (*S1* to *S10*)

hydroxy 2-butanone (or acetoin) were the main ketones identified in Pategrás cheeses. In eight of the analyzed samples, acetoin was the most abundant ketone, accounting for 50% of the total ketones. Diacetyl and its reduction product, acetoin, have been reported as odor impact compounds in Gruyère Switzerland cheese (Mallia et al. 2005). They are produced by certain microorganisms such as citrate-positive lactic acid bacteria and mesophilic lactobacilli through different pathways that include lactose and citrate metabolism or amino acid catabolism (McSweeney and Sousa 2000).

Esters and *aldehydes* represented two minority classes of compounds. In general, their relative percentages did not exceed 8% of the total volatile profile. Acetaldehyde was the predominant aldehyde in several samples analyzed. It is produced by lactic acid bacteria from lactose fermentation, pyruvate metabolism, and by breakdown of threonine. 2- and 3-Methylbutanal were also important from a quantitative viewpoint. They are derived from branched-chain amino acid catabolism. In Gruyère and Maasdam cheeses, aldehydes were identified as important components of water-soluble fraction (Engels et al. 1997). In particular, branched-chain aldehydes are considered as characteristic compounds of cheeses made with PAB (Thierry and Maillard 2002; Thierry et al. 2004a, 2005a, b). On the other hand, ethyl esters were the main esters in Pategrás cheeses. Among them, ethyl acetate, ethyl propanoate, and ethyl butanoate were detected in all samples. Ethyl propanoate was the most abundant ester in several samples. This compound is not usually identified in the volatile profile of cheeses made without PAB. In fact, ethyl propanoate biosynthesis in cheeses with added PAB is likely due to a combined effect of factors such as the presence of substrates (propanoic acid and ethanol), enzymes, and environment conditions (Richoux et al. 2008). The role of alcohols, mainly ethanol, on ester biosynthesis in cheeses has been extensively investigated (Liu et al. 2004; Thierry et al. 2006; Richoux et al. 2008). Data on the volatiles in various cheeses suggest that alcohol availability is the limiting factor of ester biosynthesis and that the levels of esters seemed to be directly related to the concentrations of the corresponding alcohols. Moreover, Thierry et al. (2006) demonstrated that there was not a direct relationship between the concentration of acids and that of the corresponding ethyl ester.

3.2.2 Pategrás cheeses made at pilot scale

Thirty-four compounds were detected in the volatile profile of Pategrás cheeses produced at pilot scale during ripening: 4 aldehydes, 8 ketones, 3 esters, 8 alcohols, and 11 acids. The majority of them were common in both *R* and *E* cheeses, but they differed quantitatively. The levels of a great number of volatile compounds were significantly influenced by the presence of PAB. Figures 2, 3, 4, 5, and 6 show the evolution of the volatile compounds, grouped by chemical families, throughout the ripening process (35, 45, and 60 days) for both types of cheeses.

Aldehydes Among this group, acetaldehyde was the most abundant aldehyde identified at all stages (Fig. 2). At 35 and 45 days of ripening, cheeses *E* had a significantly higher level than cheeses *R* ($P<0.05$) whereas at the end of ripening, similar quantities were detected ($P>0.05$). The evolution of acetaldehyde content throughout the ripening period showed a marked increase after 45 days. Data reported on acetaldehyde level in cheeses with PAB and its variations are scarce, which is mainly associated with poor

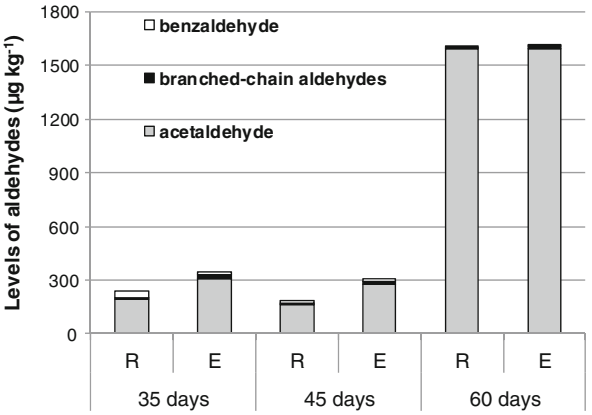

Fig. 2 Levels of aldehydes in cheeses made with and without added PAB (*E* and *R*, respectively) at 35, 45, and 60 days of ripening

analytical techniques for its extraction. Some studies indicate that certain strains of *Propionibacterium* can synthesize acetaldehyde as well as reduce it to the corresponding primary alcohols (Keenan and Bills 1968). Among branched-chain aldehydes, 3-methylbutanal was detected in all cheeses whereas 2-methylbutanal was only found in those made with PAB. They decreased in cheeses *E* or did not show important variations in cheeses *R* during ripening. Aldehydes are considered as transitory compounds in cheeses since they are quickly transformed to alcohols or to acids. The results in Figs. 4 and 5 suggest that branched-chain aldehydes from cheeses *E* could have been converted to the corresponding branched-chain alcohols or branched-chain acids. Throughout the maturation period, cheeses *E* had significantly higher amounts ($P < 0.05$) of branched-chain aldehydes than cheeses *R*. This finding is not surprising since it is well documented that PAB can enhance the production of branched-chain compounds, mainly those isoleucine-derived products such as 2-methylbutanal (Thierry et al. 2004a, 2005a, b). Benzaldehyde was identified at all stages. Its level decreased in both types of cheeses, but this decrease was more pronounced in cheeses *R*. At the end of ripening, cheeses

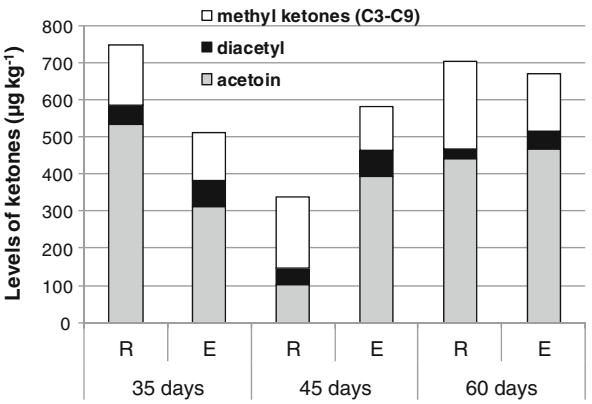

Fig. 3 Levels of ketones in cheeses made with and without added PAB (*E* and *R*, respectively) at 35, 45, and 60 days of ripening

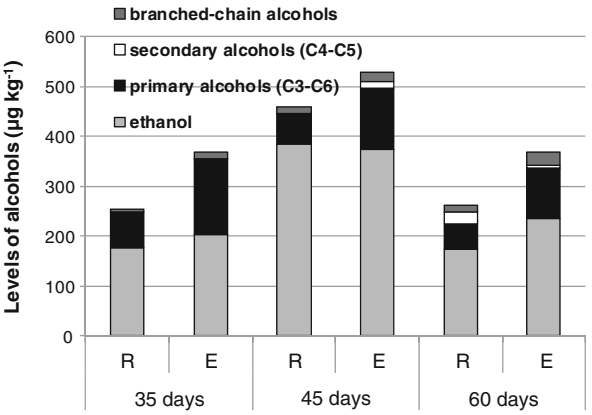

Fig. 4 Levels of alcohols in cheeses made with and without added PAB (*E* and *R*, respectively) at 35, 45, and 60 days of ripening

with added PAB reached values significantly higher in comparison to the reference cheeses ($P < 0.05$). *P. freudenreichii* has demonstrated the ability to produce various aromatic compounds from phenylalanine in in vitro assays (Thierry and Maillard 2002). However, studies carried out in cheeses indicated that benzaldehyde production was not significantly affected by PAB (Thierry et al. 1999).

Ketones Acetoin was the most abundant ketone identified (Fig. 3). The behavior of acetoin content during ripening differed in both types of cheeses. A steady increase in acetoin level over the entire ripening period was observed in cheeses made with added PAB, whereas a marked decrease until 45 days, and then a strong increase up to 60 days was recorded in cheeses *R*. The comparison of acetoin content between cheeses with and without PAB at each sampling time revealed that the levels were statistically higher in cheeses *R* than in cheeses *E* at 35 days ($P < 0.05$), whereas cheeses *E* had higher levels than their counterparts without PAB at 45 days ($P < 0.05$). From a quantitative

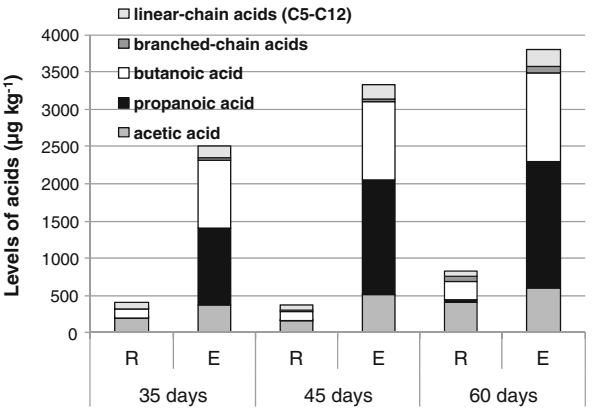

Fig. 5 Levels of acids in cheeses made with and without added PAB (*E* and *R*, respectively) at 35, 45, and 60 days of ripening

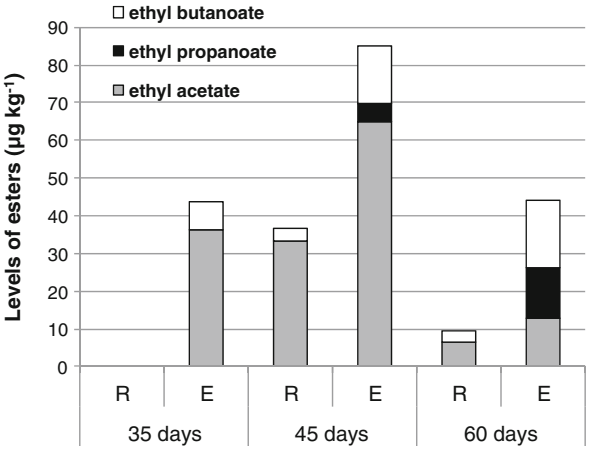

Fig. 6 Levels of esters in cheeses made with and without added PAB (*E* and *R*, respectively) at 35, 45, and 60 days of ripening

viewpoint, other important ketones were diacetyl and some methyl ketones such as 2-propanone, 2-pentanone, and 2-heptanone. Diacetyl, a compound metabolically related to acetoin, had higher levels in cheeses *E* compared to cheeses *R* at 45 and 60 days of ripening. In both types of cheeses, a steady decrease was recorded after 35 days, which was in accordance with the progressive increase of acetoin levels. Contrary to that observed in this study, a lower concentration of diacetyl in the presence of PAB (Thierry et al. 2005b) and inconsistent variations during ripening (Thierry et al. 1999) have been reported. With some exceptions, methyl ketones (C_3 to C_9) showed a moderate increase during ripening and the levels of some of them such as 2-propanone and 2-pentanone were always higher in cheeses *R* in comparison to cheeses *E*. Similarly, Thierry et al. (1999) reported increased levels of methyl ketones throughout the ripening in Emmental cheeses. Nevertheless, the role of PAB in their production was not clearly established (Thierry et al. 2005a, b).

Alcohols Ethanol was by far the most abundant alcohol detected in all cheeses (Fig. 4). As regards the evolution of ethanol during ripening, a similar behavior was observed in both types of cheeses; its level increased at 45 days and then underwent a marked decline. Besides, there were not significant differences among samples (*R* and *E*) at each sampling time ($P > 0.05$). Similarly, the amounts of ethanol found in Raclette and Swiss cheeses were similar in the presence or absence of PAB (Thierry et al. 2005a, b, 2006). Other aliphatic primary alcohols identified in the volatile profile of cheeses were 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol. Regardless of the ripening stage, 1-propanol and 1-butanol reached the highest levels in cheeses with added PAB whereas 1-hexanol was found at highest levels in cheeses *R*. Overall, they evidenced a decrease in both types of cheeses during ripening. Results reported for aliphatic alcohols in Swiss-type cheeses during ripening showed either an increase or no significant variation (Thierry et al. 2005a, b). The effect of PAB on primary alcohol biosynthesis has not yet been elucidated. Among branched-chain alcohols, only 3-methyl-1-butanol was detected and its level increased throughout the ripening period. Regardless of

the ripening time, cheeses *E* had higher amounts of this branched-chain alcohol than cheeses *R*. As mentioned, PAB have been associated with the production of branched-chain compounds. However, this ability has been shown to be highly strain dependent. This would explain the fact that 3-methyl-1-butanol was found at significantly greater levels in some cheeses made with PAB (Thierry and Maillard 2002; Thierry et al. 2005a), while in other cheeses, its concentration was not affected by the presence of these microorganisms (Thierry et al. 2005b).

Acids Eleven acids were identified in cheese samples. In general, they belonged to linear-chain fatty acids from C_2 to C_{12} and branched-chain fatty acids. Overall, at all stages of ripening assayed, the amounts of free fatty acids were higher in cheeses *E* than in cheeses *R* (Fig. 5). An important increase in the levels of the major acids in both types of cheeses during ripening was also observed. From a quantitative viewpoint, the acidic fraction of cheeses made with added PAB was mainly characterized by the presence of short-chain fatty acids: acetic, propanoic, and butanoic acids. Among them, propanoic acid was detected at the highest levels, followed by butanoic and then by acetic acid. Instead, acetic and butanoic were more prevalent in cheeses *R*. Linear-chain fatty acids from C_5 to C_{12} and branched-chain fatty acids constituted a minority group. The occurrence of acidic fraction and its evolution during ripening has been largely investigated in Swiss cheeses. Besides, in Emmental and other Swiss-type cheese varieties, acetic production was stoichiometrically related to that of propanoic acid in the ratios 1:2 by the propionic fermentation of lactic acid. This proportion was verified in Raclette cheeses (Thierry et al. 2005a). In the Pategrás cheese, the level of propanoic acid was approximately threefold higher than that of acetic acid. The results obtained in the present work about a higher production of fatty acids in cheeses *E*, derived from both lipolysis and branched-chain amino acid metabolism by PAB, were in accordance with those reported by other authors (Thierry et al. 2004a, 2005a, b).

Esters Only three ethyl esters were identified in cheese samples: ethyl acetate, ethyl propanoate, and ethyl butanoate. In general, levels of esters were significantly higher ($P < 0.05$) in cheeses *E* than in cheeses *R* (Fig. 6). At 35 days old, they were not detected in those cheeses without PAB. Ethyl propanoate was only identified in *E* cheeses. A similar trend was observed for ethyl butanoate and ethyl propanoate throughout the ripening period evaluated, showing in both compounds an important increase in cheeses *E*. Ethyl acetate had an erratic behavior; at 45 days, its level increased whereas at 60 days showed a marked decrease in both types of cheeses. The ability of enzymes apported by PAB for synthesizing esters is well known. In particular, the production seems to increase during ripening of cheeses in a warm room (Liu et al. 2004). Biosynthesis of ethyl esters is also dependent on the level of free fatty acids and ethanol (Thierry et al. 1999, 2006). In our study, the highest levels of ethyl esters observed in cheeses *E* could be attributed, at least partially, to a higher availability of the corresponding acids in comparison to cheeses *R*. On the other hand, the decrease in the quantities of ethyl acetate in both cheeses at 60 days of ripening could be associated to the decline observed in the ethanol levels (Fig. 4). Besides, ester accumulation is determined by a delicate balance between ester synthesis and ester hydrolysis. In fact, the decrease in the concentration of certain esters observed

during ripening of some cheese varieties has been attributed to the hydrolysis of esters (Liu et al. 2004).

4 Conclusions

This preliminary study contributes to a deeper knowledge of the ripening process of Pategrás cheese. The high variability observed in some physicochemical parameters, mainly ripening degree, could be attributed to a lack of standardized cheese-making processes. Although microbiological analysis was not performed, this study shows that *P. freudenreichii* PS-1 was capable of growth in a semi-hard cheese as Pategrás and modifies the volatile fraction during ripening. This statement is based on previous studies of viability of this strain in Pategrás cheese. Besides, the presence of characteristic eyes and propanoic acid at high levels are considered as index of growth and fermentation. The inclusion of PAB affected substantially the volatile profile; their presence increased the levels of several compounds, such as branched-chain aldehydes, primary alcohols, branched-chain alcohols, diacetyl, acetoin, ethyl esters, branched-chain acids, and short-chain acids. Complementary sensory and microbial analysis should be made in the future to go further into the relationship between these parameters and aroma bioformation, which would allow a more complete characterization of Pategrás cheese.

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