Journal of Food Protection, Vol. 68, No. 7, 2005, Pages 1399–1407

Production of Volatile Compounds in Cheese by *Pseudomonas fragi* Strains of Dairy Origin

PILAR MORALES, ESTRELLA FERNÁNDEZ-GARCÍA, AND MANUEL NUÑEZ*

Departamento de Tecnología de Alimentos, INIA, Carretera de La Coruña Km 7, Madrid, 28040 Spain

MS 04-562: Received 15 December 2004/Accepted 4 February 2005

ABSTRACT

Volatile compounds produced in cheese by five *Pseudomonas fragi* strains isolated from 1-day-old raw milk cheeses were investigated. Each strain was representative of a different biochemical group of isolates of identical phenotypic characteristics, according to identification with API 20 NE strips. The five strains were ascribed to the species *P. fragi* after 16S rRNA sequencing because of their high degree of coincidence with *P. fragi* ATCC 4973. In each of two experiments, carried out on different days, five cheeses were made at laboratory scale from pasteurized milk separately inoculated with approximately 10^5 CFU/ml of each *P. fragi* strain. After 12 days at 10°C, mean counts of *P. fragi* strains were close to 10^{10} CFU/g in the outer part of cheeses and close to 10^8 CFU/g in the inner part. A total of 131 volatile compounds, 49 of which were further characterized, were identified in cheeses by gas chromatography–mass spectrometry after extraction with a purge and trap apparatus. Abundances of compounds were generally higher in the outer part of cheeses. Production of volatile compounds was clearly strain dependent. Only two strains produced ethyl esters, and three produced nonethyl esters. Ethyl acetate, ethyl butyrate, ethyl caproate, methyl acetate, isopropyl acetate, and propyl tiglate were the major esters, and ethanol, 2-propanol, and 3-methyl butanol were the major alcohols. Undecene was the major hydrocarbon, dimethyl sulfide and methyl thiocyanate the major sulfur compounds, and 2-pentanone the major ketone. Two aromatic compounds, styrene and *o*-dichlorobenzene, were present in all cheeses.

Pseudomonas is the most important genus of psychrotrophic microorganisms associated with the spoilage of milk and dairy products *(29)*. Within *Pseudomonas*, *P. fragi*, *P. fluorescens*, and *P. putida* are the three species of greatest concern *(22, 25)*. Inadequately disinfected milking equipment is the main source of *Pseudomonas* and other psychrotrophic bacteria that become predominant during refrigerated storage of raw milk *(4)*, and improperly cleaned pasteurizers and filling machines are the most common sources of postpasteurization contamination *(10)*.

Proteinases from *Pseudomonas* have been related to textural changes in milk, such as gelation and increased viscosity, and to unclean and bitter flavors in cheese and other dairy products *(8)*, whereas their lipases and esterases have been associated to rancid and fruity aromas *(24)*. Populations of *Pseudomonas* range from 5×10^6 to 2×10^7 CFU/ml at the time a change in milk flavor is perceived *(4)*. Also, most evidence suggests that defects in cheese flavor or texture are detected when psychrotroph counts in raw milk exceed 10^6 CFU/ml *(2, 4, 5, 27)*.

Pseudomonas species produce different volatile compounds during growth in milk. In particular, *P. fragi* has been reported to produce high levels of short-chain fatty acid ethyl esters, which are responsible for the fruity flavor defect *(23)*. The combination of gas chromatography–mass spectrometry with olfactory analysis allowed the identification of ethyl butyrate, ethyl 3-methylbutanoate, and ethyl hexanoate as the compounds responsible for the strawberry-like odor of *P. fragi* milk cultures *(3)*. Differences between the volatile compound profiles of five bacterial strains belonging to the species *P. fragi*, *P. fluorescens*, *Bacillus subtilis*, *Enterobacter aerogenes*, and *Lactococcus lactis* were observed when grown in milk *(12)*. Descriptive aroma analysis of milk cultures of two strains of each of the species *P. fragi*, *P. fluorescens*, and *P. putida* concluded that sensory aroma characteristics of milk cultures were strain dependent *(11)*.

Microbiota and native enzymes present in raw milk are considered of crucial importance for the development in raw milk cheeses of flavor notes lacking in pasteurized milk cheeses. Although the effects of psychrotrophs on milk coagulation characteristics and on proteolysis and lipolysis during cheese ripening are well known *(4, 8, 24)*, our knowledge of the effects of *Pseudomonas* and other psychrotrophic bacteria on the volatile compound profile of cheese remains scarce. The objective of this work was to investigate the production of volatile compounds by different strains of dairy origin belonging to the species *P. fragi* in cheeses manufactured from milk inoculated separately with each strain.

MATERIALS AND METHODS

Strain isolation and identification. Strains were isolated from ten 1-day-old raw ewes' milk cheeses from five different dairies in central Spain. Cheese homogenates in sterile 20 g/liter sodium citrate solution were serially diluted in sterile 1 g/liter peptone solution and plated on PMK agar (Biolife, Milano, Italy).

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* Author for correspondence. Tel: 34-91-3476799; Fax: 34-91-3572293; E-mail: nunez@inia.es.

Ten colonies per plate were picked randomly and examined for Gram stain, catalase test, and oxidase test. Five gram-negative, catalase-positive, oxidase-positive rods from each of the 10 cheeses were grouped into different biochemical profiles with the aid of the API 20 NE system (bioMérieux, Marcy l'Etoile, France).

One isolate representative of each biochemical profile was selected and identified by 16S rRNA sequencing, basically as described by Wiedmann et al. *(30).* Lysozyme plus proteinase K lysates were serially diluted, and 1 μl of the 1:1,000 dilution was used for PCR amplification. Partial sequencing of the amplified fragment (approximately 1.1 kb) was carried out, and the obtained sequence was compared by BLAST search analysis with sequences in the National Center for Biotechnology Information (NCBI) database. Each strain was assigned to the species showing the highest degree of similarity after comparison with type strains of *Pseudomonadaceae* from different collections. All the strains were maintained at −80°C in nutrient broth with 150 g/liter glycerol added.

Cheese manufacture and sampling. Cheeses were made at laboratory scale in duplicate experiments carried out on different days. Each experiment consisted of six 4-liter vats of pasteurized (78°C for 15 s) cow's milk (total viable counts below 5×10^2 CFU/ml). Milk in five vats was separately inoculated with each of the five selected *P. fragi* strains. Milk in the sixth vat was not inoculated with any of the *P. fragi* strains, and 250 μg/ml amoxicillin and 62.5 μg/ml clavulanic acid were added to milk to prevent bacterial growth. Curds from the sixth vat were pressed for 2 h and held at −40°C until analysis. The manufacturing procedure was that of Hispánico cheese, a semihard Spanish variety, except that glucono-δ-lactone (Chr. Hansen, Madrid, Spain) was used instead of lactic starter cultures for milk and curd acidification to circumvent interferences by the metabolism of lactic acid bacteria. Milk at 31°C, with 0.1 g/liter $CaCl_2$ and 10 g/liter glucono-δ-lactone added, was inoculated with 2 ml of a fresh culture of the respective *P. fragi* strain in sterile milk to yield approximately 10^5 CFU/ml. After 20 min, 2.66 ml of a fresh 2% dilution of Maxiren 150 rennet (Gist Brocades, Delft, The Netherlands) was added, and milk was held at 31°C for 40 min to coagulate. The curd was cut to rice grain size, heated to 38°C, held for 15 min at this temperature to favor whey expulsion, and transferred into cylindrical molds. Two cheeses, approximately 250 g in weight, were obtained per vat. They were pressed overnight at 20°C and 0.7 kg/cm^2 pressure. Next morning, cheeses were salted for 20 min in a 150 g/liter NaCl solution at 20°C and left to ripen at 10°C.

Curds from inoculated milk were sampled after 2 h in press for microbiological analysis. Control curds from milk not inoculated with *P. fragi* were sampled after 2 h in press to determine the initial levels of volatile compounds in each experiment. Differential sampling of cheeses was carried out in the outer part, at 5 mm depth, and in the inner part, consisting of the rest of the cheese, after 6 or 12 days of ripening. Samples for volatile compound analysis were wrapped in aluminum foil, vacuum packed, and kept at −40°C.

Microbiological analysis and cheese pH. Curd and cheese samples were homogenized in sterile 20 g/liter sodium citrate solution with a homogenizer (IUL, Barcelona, Spain). Decimal dilutions were prepared in sterile 1 g/liter peptone solution and spread plated in duplicate with a DS Plus spiral plater (Interscience, Saint-Nom-La-Bretèche, France). PMK agar plates incubated at 30°C for 24 h were used for the determination of *P. fragi* counts; plate count agar (PCA; Oxoid, Basingstoke, UK) plus 1 g/liter glucose was used to check for homogeneity of colonies and absence of contaminants; VRBGA (VRBA [Oxoid] with 10 g/liter glucose added) was used to check for absence of *Enterobacteriaceae;* and MRS (Biolife) pH 5.7 was used to check for absence of lactic acid bacteria.

Cheese pH was measured in duplicate with a penetration electrode (Xerolyt 52-32, Crison, Barcelona, Spain).

Volatile compound analysis. Duplicate cheese samples (10 g) were homogenized in an analytical blender with 20 g of anhydrous Na_2SO_4 and 30 μl of an internal standard aqueous solution containing 0.84 mg/ml cyclohexanone and 0.51 mg/ml camphor (Sigma-Aldrich Química, Alcobendas, Spain). An aliquot (2.25 g) of the mixture was subjected to dynamic headspace with helium (45 ml/min) in an automatic HP 7695 purge and trap apparatus (Hewlett-Packard, Palo Alto, Calif.) at 50°C for 15 min, with 10 min of previous equilibrium. Volatile compounds were concentrated in a Tenax trap maintained at 30°C and 6.5 psi back pressure, with 1 min of dry purge, and desorbed during 1 min at 230°C directly into the injection port at 220°C, with a split ratio of 1:20, and 1.4 ml/min He flow.

Gas chromatography was carried out in an HP-6890 gas chromatography–mass spectrometry apparatus equipped with a capillary column HP Innowax (60 m long, 0.25 mm outside diameter, 0.5 μm film thickness). Chromatographic conditions were 12.5 min at 45°C, 4°C/min to 114°C, 6 min at 114°C, 7°C/min to 143°C, 15°C/min to 240°C, 4 min at 240°C, and He flow at 1 ml/min. Total analysis time was 51 min. Detection was performed with the mass spectrometer operating in the scan mode, 2.6 scans per s, with ionization energy of 70 eV and source and quadrupole temperatures of 230°C and 150°C, respectively. Peak identification was by comparison of retention times and ion spectra from real standards (Sigma-Aldrich Química) and spectra from the Wiley 275 library (Wiley & Sons Inc., New York). For each compound, including internal standards, the sum of the areas of the peaks of up to four characteristic ions was obtained. Only compounds with abundance values above 20,000 in at least one cheese sample were further considered. The relative abundance of a particular compound was calculated as 1,000 times the sum of the areas of the peaks of its characteristic ions divided by the sum of the areas of the peaks of the characteristic ions of cyclohexanone.

Statistical analysis. Analysis of variance was carried out on pH values, log counts, and relative abundances of seven groups of volatile compounds (ethyl esters, non–ethyl esters, alcohols, alkanes, sulfur compounds, ketones, and aromatic compounds), with *P. fragi* strain and cheese sample as main effects, by means of the SPSS Win 8.0 program. Also, the relative abundances of 49 individual volatile compounds in cheeses made with each of the *P. fragi* strains were subjected to one-way analysis of variance with bacterial strain as main effect, with data from 6-day-old and 12-day-old cheeses treated separately. Comparison of means was carried out by Tukey's test.

RESULTS

Fifty *Pseudomonas* spp. isolates from the raw milk cheeses were grouped into 12 different biochemical profiles with the aid of the API 20 NE system. One isolate per biochemical profile was further characterized by comparing its 16S rRNA sequence with the sequences of different *Pseudomonadaceae* collection strains. Five of the 12 isolates were ascribed to the species *P. fragi,* by far the most abundant species. The rest of the isolates were two *P. libanensis,* one *P. brenneri,* one *P. graminis,* one *P. lundensis,* one *P. putida,* and one *P. rhodesiae.* The coincidence

TABLE 1. *Counts of* Pseudomonas fragi *and pH values during cheese manufacture and ripening*[a]

	P. fragi (log CFU/g)	pH
Milk	4.86 ± 0.34 A	6.74 ± 0.05 A
2-h curds	6.63 ± 0.47 B	5.26 ± 0.09 B
6-d-old cheese outer samples	9.64 ± 0.09 E	5.50 ± 0.06 D
6-d-old cheese inner samples	7.68 ± 0.33 C	5.42 ± 0.04 C
12-d-old cheese outer samples	9.87 ± 0.10 F	5.39 ± 0.07 C
12-d-old cheese inner samples	8.13 ± 0.31 D	5.43 ± 0.02 CD

[a] Mean values for the five stains from duplicate experiments. Means in the same column not followed by the same letter are significantly different ($P < 0.05$).

of the nucleotide sequences of *P. fragi* strains with the nucleotide sequence of type strain *P. fragi* ATCC 4973 ranged from 99.1% for strain *P. fragi* 26 to 99.7% for strain *P. fragi* 37. These five *P. fragi* strains were used in cheesemaking experiments.

Bacterial counts and cheese pH. The mean count of the five *P. fragi* strains in inoculated milk was 4.86 log CFU/ml, whereas their mean count in 2-h curds reached 6.63 log CFU/g (Table 1). Counts of the five *P. fragi* strains increased from milk to curds and to cheese at 6 and 12 days of ripening. Higher counts were found for all strains in the outer part of cheeses than in the inner part (Table 1), with no significant differences between *P. fragi* strains during cheese manufacture or ripening.

A low pH (mean 5.26; Table 1) was recorded for curds after 2 h in press because of a rapid acidification of the substrate caused by the hydrolysis of glucono-δ-lactone. Values of pH in the inner and outer parts of cheeses after 6 days of ripening were significantly higher than the pH of 2-h curds. A slight decrease in pH value was observed from day 6 to day 12 in the outer part of cheeses.

Volatile compounds. Gas chromatography–mass spectrometry analysis of the volatile fraction of cheeses made from milk inoculated with single strains of *P. fragi* detected 131 different compounds, 96 of which were already present in 2-h curds made from milk not inoculated with *P. fragi* strains. The number of compounds exhibiting abundance values above 20,000 in at least one cheese sample was 103. Among them, 22 compounds already present in 2-h curds were found to decline significantly ($P < 0.05$) as cheese aged. The levels of 32 other compounds did not vary significantly during ripening in any of the cheeses made from milk inoculated with *P. fragi* strains. Analysis of variance permitted the selection of 49 volatile compounds for which concentrations increased significantly ($P < 0.05$) with cheese age for at least one of five *P. fragi* strains inoculated into milk. They were grouped into 13 ethyl esters, 12 non–ethyl esters, 10 alcohols, six hydrocarbons, four sulfur compounds, two ketones, and two aromatic compounds (Table 2).

Volatile compounds were mostly produced during the first 6 days of ripening. Only the levels of total sulfur compounds and total aromatic compounds continued to increase from day 6 to day 12 (Table 2). The levels of ethyl esters, hydrocarbons, sulfur compounds, and aromatic compounds were significantly ($P < 0.05$) higher in the outer part than in the inner part of 12-day-old cheeses (Table 2).

Esters. Production of ethyl esters in cheeses manufactured from milk inoculated with single strains of *P. fragi* is shown in Table 3. Thirteen different ethyl esters were found in *P. fragi* 39 cheeses, and 10 ethyl esters in *P. fragi* 21 cheeses, which were the strongest producing strains. Ethyl ethanoate accounted for 88 and 43%, respectively, of total ethyl esters produced in the inner part of 12-day-old *P. fragi* 39 and *P. fragi* 21 cheeses and for 85 and 28%, respectively, of total ethyl esters in the outer part. Ethyl butanoate and ethyl hexanoate were also of considerable importance in *P. fragi* 39 cheeses. The concentration of total ethyl esters in the outer part of 12-day-old cheeses was 2.7-fold that in the inner part for *P. fragi* 21 and 5.1-fold that for *P. fragi* 39. Small amounts of some ethyl esters were present in *P. fragi* 4, *P. fragi* 26, and *P. fragi* 37 cheeses, although their relative abundances did not differ significantly from those found in 2-h curds.

As in the case of ethyl esters, *P. fragi* 21 and *P. fragi* 39 were the strains producing both the largest number of different non–ethyl esters and the highest total concentra-

TABLE 2. *Levels of the main groups of volatile compounds in 2-h control curds and during ripening of cheeses made from milk inoculated separately with five* Pseudomonas fragi *strains*[a]

	2-h control curds	6-d-old cheese outer samples	12-d-old cheese outer samples	12-d-old cheese inner samples
Ethyl esters ($n = 13$)	1.64 A	224.87 B	302.63 B	79.60 A
Non–ethyl esters ($n = 12$)	0.78 A	70.88 B	67.45 B	36.05 AB
Alcohols ($n = 10$)	109.40 A	186.62 B	201.88 B	147.23 AB
Hydrocarbons ($n = 6$)	5.57 A	35.97 AB	72.63 B	20.93 A
Sulfur compounds ($n = 4$)	2.45 A	8.10 B	11.17 C	7.28 B
Ketones ($n = 2$)	1.14 A	3.48 B	3.02 B	3.28 B
Aromatic compounds ($n = 2$)	1.45 A	3.61 B	6.38 C	1.11 A

[a] Mean values of relative abundances for the five strains from duplicate experiments. Relative abundance of a compound was calculated as 1,000 times the sum of the areas of the peaks of its characteristic ions divided by the sum of the areas of the peaks of the characteristic ions of the internal standard cyclohexanone. Means in the same row not followed by the same letter are significantly different ($P < 0.05$).

TABLE 3. *Levels of ethyl esters in 2-h control curds and during ripening of cheeses made from milk inoculated separately with five* Pseudomonas fragi *strains*[a]

Volatile compound	Sample	*P. fragi* strain:					Curds
		4	21	26	37	39	
Ethyl ethanoate	6 d outer	0.37 A	291.09 B	3.81 A	0.44 A	241.98 B	0.69
	12 d outer	0.00 A	466.01 C	1.65 A	0.02 A	271.78 B	
	12 d inner	0.44 A	179.27 C	2.13 A	0.97 A	80.72 B	
Ethyl propanoate	6 d outer	0.00 A	12.05 B	0.00 A	0.00 A	18.32 C	0.00
	12 d outer	0.00 A	27.71 B	0.00 A	0.00 A	30.89 B	
	12 d inner	0.00 A	11.38 B	0.00 A	0.00 A	12.94 B	
Ethyl 2-methyl propanoate	6 d outer	0.00 A	1.91 A	0.00 A	0.00 A	6.90 B	0.00
	12 d outer	0.00 A	7.17 B	0.00 A	0.00 A	15.29 C	
	12 d inner	0.00 A	0.99 AB	0.00 A	0.00 A	2.90 B	
Ethyl butanoate	6 d outer	1.05 A	18.26 B	2.50 A	0.75 A	116.31 C	0.69
	12 d outer	0.49 A	24.09 A	1.23 A	0.81 A	103.09 B	
	12 d inner	2.03 A	9.93 A	0.62 A	0.61 A	36.69 B	
Ethyl 2-methyl butyrate	6 d outer	0.00 A	0.00 A	0.00 A	0.00 A	6.35 B	0.00
	12 d outer	0.00 A	1.84 A	0.00 A	0.00 A	14.07 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	1.27 B	
Ethyl 3-methyl butyrate	6 d outer	0.00 A	1.01 A	0.00 A	0.00 A	28.90 B	0.02
	12 d outer	0.00 A	3.76 A	1.12 A	0.15 A	79.49 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.24 A	8.71 B	
Ethyl pentanoate	6 d outer	0.00 A	0.18 A	0.00 A	0.00 A	9.09 B	0.00
	12 d outer	0.00 A	0.47 A	0.00 A	0.00 A	11.63 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	1.28 B	
Ethyl 2-butenoate	6 d outer	0.00 A	2.72 A	1.01 A	0.00 A	22.34 B	0.00
	12 d outer	0.00 A	1.91 A	0.63 A	0.00 A	18.86 B	
	12 d inner	0.00 A	0.55 A	0.00 A	0.00 A	6.29 B	
Ethyl hexanoate	6 d outer	0.25 A	5.74 A	0.11 A	0.26 A	292.26 B	0.24
	12 d outer	0.13 A	8.01 A	0.19 A	1.02 A	366.26 B	
	12 d inner	0.61 A	0.45 A	0.00 A	0.00 A	33.91 B	
Ethyl 2-methyl 2-butenoate	6 d outer	0.00 A	0.00 A	0.00 A	0.00 A	6.47 B	0.00
	12 d outer	0.00 A	0.00 A	0.00 A	0.00 A	16.04 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	1.25 B	
Ethyl heptanoate	6 d outer	0.00 A	0.00 A	0.00 A	0.00 A	6.03 B	0.00
	12 d outer	0.00 A	0.00 A	0.00 A	0.00 A	8.78 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	0.17 B	
Ethyl 2-hexenoate	6 d outer	0.00 A	0.00 A	0.00 A	0.00 A	8.33 B	0.00
	12 d outer	0.00 A	0.00 A	0.00 A	0.00 A	7.88 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	0.72 B	
Ethyl octanoate	6 d outer	0.00 A	4.65 B	0.00 A	0.00 A	12.89 C	0.00
	12 d outer	0.00 A	5.91 B	0.00 A	0.00 A	14.81 C	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	0.94 B	
Total ethyl esters	6 d outer	1.67 A	337.62 B	7.44 A	1.44 A	776.16 C	1.64
	12 d outer	0.62 A	546.88 B	4.82 A	2.00 A	958.86 C	
	12 d inner	3.08 A	202.57 B	2.75 A	1.82 A	187.77 B	

[a] Mean values of relative abundances (calculated as indicated in Table 2 footnote) from duplicate experiments. Means in the same row not followed by the same letter are significantly different ($P < 0.05$).

tions of these compounds (Table 4). Methyl ethanoate, 2-propyl ethanoate, and 4-penten-1-ol ethanoate were the most abundant non–ethyl esters in *P. fragi* 21 cheeses; methyl ethanoate, methyl hexanoate, and propyl 2-methyl 2-butenoate were the most abundant in *P. fragi* 39 cheeses. The concentration of total non–ethyl esters in the outer part of 12-day-old cheeses was 1.2-fold that in the inner part for *P. fragi* 21 and 4.3-fold that for *P. fragi* 39. Strain *P. fragi* 26, which was not a strong producer of ethyl esters, produced however considerable amounts of non–ethyl esters such as isopropyl ethanoate, methyl ethanoate, methyl propanoate, and methyl 3-methyl-butanoate. *P. fragi* 4 and *P. fragi* 37 cheeses did not contain significant amounts of non–ethyl esters.

Alcohols. Relative abundances of alcohols produced in cheeses by *P. fragi* strains are shown in Table 5. Considerable levels of 2-propanol and ethanol were already present in 2-h curds. Differences between strains in the levels of alcohols were less marked than in the case of esters, in

TABLE 4. *Levels of non–ethyl esters in 2-h control curds and during ripening of cheeses made from milk inoculated separately with five* Pseudomonas fragi *strains*[a]

		P. fragi strain:					
Volatile compound	Sample	4	21	26	37	39	Curds
Methyl ethanoate	6 d outer	1.17 A	85.37 C	8.82 A	0.91 A	32.35 B	0.63
	12 d outer	0.82 A	71.34 B	8.60 A	0.79 A	14.93 A	
	12 d inner	1.06 A	67.94 C	9.41 AB	0.63 A	15.85 B	
Methyl propanoate	6 d outer	0.00 A	2.20 B	0.55 A	0.00 A	2.46 B	0.02
	12 d outer	0.00 A	3.57 C	1.96 B	0.00 A	1.81 B	
	12 d inner	0.00 A	3.29 C	1.51 B	0.00 A	1.86 B	
Methyl butanoate	6 d outer	0.00 A	0.00 A	0.57 A	0.00 A	9.72 B	0.08
	12 d outer	0.00 A	0.00 A	0.79 A	0.00 A	4.72 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	4.00 B	
Methyl 3-methyl butanoate	6 d outer	0.00 A	0.00 A	0.00 A	0.00 A	7.20 B	0.00
	12 d outer	0.00 A	0.00 A	3.80 B	0.00 A	7.87 C	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	1.40 B	
Methyl hexanoate	6 d outer	0.00 A	0.10 A	0.30 A	0.00 A	37.88 B	0.04
	12 d outer	0.00 A	0.10 A	1.00 A	0.02 A	37.00 B	
	12 d inner	0.09 A	0.00 A	0.00 A	0.00 A	3.74 B	
Methyl octanoate	6 d outer	0.00 A	0.31 A	0.00 A	0.00 A	2.03 B	0.00
	12 d outer	0.00 A	0.24 A	0.00 A	0.00 A	1.90 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	
2-Propyl ethanoate	6 d outer	0.00 A	65.22 C	15.07 B	0.00 A	9.45 AB	0.00
	12 d outer	0.00 A	50.17 C	8.65 B	0.00 A	3.57 AB	
	12 d inner	0.08 A	48.40 B	7.86 A	0.00 A	3.83 A	
2-Propyl hexanoate	6 d outer	0.00 A	0.00 A	0.00 A	0.00 A	4.79 B	0.00
	12 d outer	0.00 A	0.00 A	0.00 A	0.00 A	5.13 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	
Penthyl ethanoate	6 d outer	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	0.00
	12 d outer	0.00 A	3.00 B	0.00 A	0.00 A	0.00 A	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	
4-Penten-1-ol ethanoate	6 d outer	0.00 A	5.51 B	0.00 A	0.00 A	1.50 A	0.00
	12 d outer	0.00 A	18.58 C	0.00 A	0.00 A	2.99 B	
	12 d inner	0.00 A	3.28 B	0.00 A	0.00 A	0.70 A	
Propyl 2-methyl-2-butenoate	6 d outer	0.00 A	0.00 A	0.28 A	0.00 A	60.45 B	0.00
	12 d outer	0.00 A	0.00 A	2.73 A	0.00 A	75.88 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	5.33 A	
3-Methyl 2-butenyl ethanoate	6 d outer	0.00 A	0.22 A	0.00 A	0.00 A	0.00 A	0.00
	12 d outer	0.00 A	4.38 C	0.00 A	0.00 A	0.92 B	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	
Total non–ethyl esters	6 d outer	1.17 A	158.92 B	25.59 A	0.91 A	167.83 B	0.78
	12 d outer	0.82 A	151.38 B	27.54 A	0.81 A	156.71 B	
	12 d inner	1.23 A	122.92 C	18.78 AB	0.63 A	36.71 B	

[a] Mean values of relative abundances (calculated as indicated in Table 2 footnote) from duplicate experiments. Means in the same row not followed by the same letter are significantly different ($P < 0.05$).

particular for the inner part of cheeses. The five *P. fragi* strains were responsible for increases in the relative abundance of 2-propanol during the first 6 days of ripening, up to 6.2-fold in the case of *P. fragi* 26, whereas the level of this compound decreased from day 6 to day 12 in the outer part of all cheeses. The concentration of ethanol increased during ripening of *P. fragi* 37 and *P. fragi* 39 cheeses and tended to decrease in *P. fragi* 4 and *P. fragi* 26 cheeses. Relative abundances of branched-chain alcohols such as 2-methyl propanol, 3-methyl butanol, and, to a lesser extent, 2-ethyl hexanol increased significantly ($P < 0.05$) during ripening. *P. fragi* 39 cheeses contained the highest levels of total alcohols after ripening for 12 days. The concentration of total alcohols in the outer part of 12-day-old cheeses was 2.0-fold that in the inner part for *P. fragi* 39 and only 0.8-fold that for *P. fragi* 21.

Miscellaneous compounds. Four of the six hydrocarbons, two of four sulfur compounds, one of two ketones, and two of two aromatic compounds found in cheeses were already present in 2-h curds (Table 6). Regarding hydrocarbons, *P. fragi* 26 was responsible for the highest concentrations of undecene in cheeses, whereas *P. fragi* 21 produced the highest levels of pentene, nonene, and cycloundecene and *P. fragi* 39 produced the highest levels of cyclohexane. Significantly ($P < 0.05$) higher amounts of methylcyclopentane were found in the inner part than in the outer part of 12-day-old *P. fragi* 21 and *P. fragi* 26 cheeses.

TABLE 5. *Levels of alcohols in 2-h control curds and during ripening of cheeses made from milk inoculated separately with five* Pseudomonas fragi *strains*[a]

Volatile compound	Sample	*P. fragi* strain: 4	21	26	37	39	Curds
2-Propanol	6 d outer	104.79 A	68.84 A	158.13 B	85.56 A	83.25 A	25.58
	12 d outer	58.91 AB	39.41 A	77.91 B	57.52 AB	41.91 A	
	12 d inner	53.67 A	63.50 A	110.28 B	60.13 A	54.39 A	
Ethanol	6 d outer	85.71 B	21.04 A	14.55 A	81.11 B	100.78 B	78.22
	12 d outer	35.98 AB	30.04 A	3.90 A	117.23 B	227.60 C	
	12 d inner	32.21 A	49.95 AB	17.37 A	53.56 AB	94.76 B	
2-Methyl-l-propanol	6 d outer	2.57 A	3.03 A	3.69 A	4.04 A	3.79 A	1.41
	12 d outer	9.46 A	4.78 A	9.69 A	11.56 A	25.85 B	
	12 d inner	4.78 A	2.28 A	4.86 A	4.45 A	9.06 A	
2-Pentanol	6 d outer	1.35 AB	3.02 B	0.90 A	3.13 B	2.34 AB	0.00
	12 d outer	1.73 AB	2.53 BC	1.51 A	3.36 C	1.85 AB	
	12 d inner	0.46 A	2.12 B	0.80 AB	2.13 B	2.02 B	
3-Methyl-l-butanol	6 d outer	4.69 A	3.92 A	5.40 A	4.57 A	6.69 A	0.39
	12 d outer	19.98 A	11.16 A	40.91 B	24.25 A	45.86 B	
	12 d inner	12.09 BC	4.33 A	13.12 BC	9.63 B	16.64 C	
3-Methyl 3-butenol	6 d outer	6.63 C	2.07 A	7.20 C	3.05 AB	4.00 B	0.00
	12 d outer	7.19 C	0.73 A	10.12 D	3.35 B	4.39 B	
	12 d inner	6.82 C	0.00 A	8.13 D	3.20 B	2.74 B	
2-Heptanol	6 d outer	0.08 A	0.36 AB	0.45 B	0.05 A	0.10 AB	0.05
	12 d outer	0.78 B	0.73 AB	0.50 AB	0.32 A	0.65 AB	
	12 d inner	0.03 A	0.05 A	0.00 A	0.08 A	0.16 A	
3-Methyl-2-butenol	6 d outer	1.12 B	0.00 A	0.00 A	0.21 A	0.39 A	0.00
	12 d outer	1.24 C	0.00 A	0.00 A	0.57 B	0.49 B	
	12 d inner	1.41 D	0.00 A	0.00 A	0.81 C	0.50 B	
1-Hexanol	6 d outer	2.84 A	1.10 A	0.20 A	3.71 A	8.98 B	0.06
	12 d outer	3.54 AB	1.20 A	0.63 A	6.31 B	11.68 C	
	12 d inner	2.19 BC	0.19 AB	0.00 A	3.71 C	3.95 C	
2-Ethyl-l-hexanol	6 d outer	5.04 AB	8.69 BC	4.92 AB	3.78 A	11.23 C	3.58
	12 d outer	8.13 A	8.62 A	6.63 A	6.67 A	20.07 B	
	12 d inner	2.35 A	5.49 A	4.38 A	4.58 A	6.82 A	
Total alcohols	6 d outer	214.82 B	112.07 A	195.43 AB	189.22 AB	221.54 B	109.40
	12 d outer	146.94 AB	99.20 A	151.79 AB	231.13 B	380.34 C	
	12 d inner	116.01 A	127.90 A	158.94 A	142.26 A	191.05 A	

[a] Mean values of relative abundances (calculated as indicated in Table 2 footnote) from duplicate experiments. Means in the same row not followed by the same letter are significantly different ($P < 0.05$).

The concentration of total hydrocarbons in the outer part of 12-day-old cheeses was 7.3-fold that in the inner part for *P. fragi* 26 and only 0.9-fold that for *P. fragi* 37.

With respect to sulfur compounds, *P. fragi* 39 was the strongest producer of methylthiol ethanoate, dimethyl disulfide, and methyl thiocyanate (Table 6). The concentration of total sulfur compounds in the outer part of 12-day-old cheeses was 2.3-fold that in the inner part for *P. fragi* 26 and 1.2-fold that for *P. fragi* 37. For ketones, *P. fragi* 21 was the strongest producer of 2-pentanone and the only producer of acetoxypropanone. The concentration of total ketones in the outer part of 12-day-old cheeses was 1.3-fold that in the inner part for *P. fragi* 21 and 0.5-fold that for *P. fragi* 37. Finally, concerning aromatic compounds, *P. fragi* 21 and *P. fragi* 37 were the strongest producers of styrene and *o*-dichlorobenzene, respectively. The concentration of total aromatic compounds in the outer part of 12-day-old cheeses was 9.3-fold that in the inner part for *P. fragi* 26 and 2.4-fold that for *P. fragi* 4.

DISCUSSION

Pseudomonas fragi is one of the psychrotrophic species of great concern regarding the spoilage of milk and dairy products *(22, 25)*. According to our results, it was clearly the predominant *Pseudomonas* species in 1-day-old raw ewes' milk cheeses. However, *P. putida*, *P. fluorescens*, and *P. fragi* had been reported as the most abundant species in raw and processed milk *(30)*. This apparent contradiction can be explained by the different origins of isolates, from raw milk cheeses with pH values close to 5 in our work, and from raw and processed milks with pH values close to neutrality in the work by Wiedmann et al. *(30)*.

The mean count of the five *P. fragi* strains used in our cheese-making experiments was 4.86 log CFU/ml in milk immediately after inoculation, a realistic number for *Pseudomonas* populations in milk for cheese production *(2, 4)*. Counts of the five *P. fragi* strains increased from milk to curds after 2 h in press by almost 2 log units (Table 1),

TABLE 6. *Levels of miscellaneous volatile compounds in 2-h control curds and during ripening of cheeses made from milk inoculated separately with five* Pseudomonas fragi *strains*[a]

		P. fragi strain:					
Volatile compound	Sample	4	21	26	37	39	Curds
Pentene	6 d outer	3.92 A	11.51 B	0.00 A	0.00 A	1.88 A	1.00
	12 d outer	2.59 B	9.34 C	0.00 A	0.00 A	1.66 AB	
	12 d inner	0.71 A	0.15 A	0.00 A	0.00 A	0.37 A	
Methylcyclopentane	6 d outer	6.04 A	1.64 A	3.72 A	8.51 A	2.36 A	4.10
	12 d outer	2.85 A	3.84 A	3.15 A	1.60 A	2.51 A	
	12 d inner	1.42 A	19.37 A	21.34 A	8.12 A	8.69 A	
Cyclohexane	6 d outer	1.34 A	1.37 A	3.71 A	12.72 AB	24.30 B	0.13
	12 d outer	1.48 A	5.77 AB	6.40 AB	9.26 AB	18.03 B	
	12 d inner	1.47 A	2.75 A	7.12 AB	7.08 AB	21.65 B	
Nonene	6 d outer	0.75 AB	1.98 B	0.00 A	0.00 A	0.34 AB	0.35
	12 d outer	0.56 A	2.92 B	2.80 B	0.00 A	0.47 A	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	
Undecene	6 d outer	10.82 AB	17.63 B	44.62 C	1.52 A	17.39 B	0.00
	12 d outer	13.10 A	18.83 A	222.95 B	3.60 A	26.36 A	
	12 d inner	0.02 A	0.00 A	4.12 B	0.00 A	0.26 A	
Cycloundecene	6 d outer	0.05 A	1.35 B	0.00 A	0.00 A	0.38 AB	0.00
	12 d outer	0.00 A	1.07 A	1.18 A	0.00 A	0.83 A	
	12 d inner	0.00 A	0.00 A	0.00 A	0.00 A	0.00 A	
Dimethyl sulfide	6 d outer	3.43 A	7.29 B	5.69 AB	2.82 A	4.57 AB	1.92
	12 d outer	5.15 A	5.40 A	4.64 A	2.43 A	5.00 A	
	12 d inner	2.49 A	3.10 A	1.78 A	1.53 A	2.70 A	
Methylthiol ethanoate	6 d outer	0.00 A	0.00 A	0.00 A	0.00 A	0.48 B	0.00
	12 d outer	0.00 A	1.40 B	0.00 A	0.00 A	2.54 C	
	12 d inner	0.00 A	0.54 A	0.00 A	0.00 A	1.39 B	
Dimethyl disulfide	6 d outer	1.63 BC	0.52 AB	0.00 A	2.37 C	2.10 C	0.53
	12 d outer	1.94 BC	1.11 AB	0.74 A	2.90 CD	3.43 D	
	12 d inner	1.91 AB	0.81 A	0.46 A	2.83 B	2.81 B	
Methyl thiocyanate	6 d outer	1.61 A	2.03 AB	1.54 A	1.14 A	3.27 B	0.00
	12 d outer	2.58 AB	4.41 C	2.05 A	3.78 BC	6.35 D	
	12 d inner	1.41 A	3.35 B	0.98 A	3.33 B	4.98 B	
2-Pentanone	6 d outer	2.89 A	5.00 B	3.54 AB	2.64 A	3.03 A	1.14
	12 d outer	1.73 AB	5.36 B	2.74 AB	1.14 A	2.13 AB	
	12 d inner	2.85 AB	4.40 B	3.09 AB	2.34 A	2.43 A	
Acetoxypropanone	6 d outer	0.00 A	0.32 B	0.00 A	0.00 A	0.00 A	0.00
	12 d outer	0.00 A	2.00 B	0.00 A	0.00 A	0.00 A	
	12 d inner	0.00 A	1.27 B	0.00 A	0.00 A	0.00 A	
Styrene	6 d outer	1.53 AB	3.03 B	1.24 A	1.25 A	1.82 AB	0.74
	12 d outer	1.55 A	5.41 B	2.14 A	2.36 A	3.39 AB	
	12 d inner	0.77 A	0.26 A	0.00 A	0.18 A	0.45 A	
o-Dichlorobenzene	6 d outer	1.07 A	1.42 AB	1.38 AB	2.89 B	2.43 AB	0.71
	12 d outer	2.49 A	2.87 A	2.52 A	4.44 A	4.72 A	
	12 d inner	0.93 A	0.89 A	0.50 A	0.78 A	0.77 A	

[a] Mean values of relative abundances (calculated as indicated in Table 2 footnote) from duplicate experiments. Means in the same row not followed by the same letter are significantly different ($P < 0.05$).

partly because of cell retention in the curds during whey drainage and partly because of bacterial growth in milk and curds during manufacture. In spite of the early decline in curd pH caused by glucono-δ-lactone, with a mean value of 5.26 in curds after 2 h in press, a considerable growth of *P. fragi* occurred during the first 6 days of ripening and, to a lesser degree, from day 6 to day 12. Growth and survival of *Pseudomonas* strains during manufacture and ripening of Manchego cheese of similar pH values has been reported *(27)*. In agreement with the aerobic metabolism of the genus *Pseudomonas*, higher counts were recorded for the five *P. fragi* strains in the outer part of cheeses than in the edible inner part.

The total number of 131 volatile compounds detected in cheeses made from milk inoculated with *P. fragi* strains was high, although it must be taken into account that 96 of those compounds were already present in 2-h curds made from milk not inoculated with any *P. fragi* strain. More than 600 volatile compounds have been identified so far in different cheese varieties *(6, 17)*. Fifty-one of the 131 compounds detected in this work were among the 76 found in cold-stored raw milk by Urbach *(28)*, 36 of which were already present in fresh raw milk. A complex profile, with approximately 90 compounds, 26 of which were odor-active, has also been reported for the volatile fraction of *P. fragi* milk cultures *(3)*. Twenty-two compounds decreased significantly ($P < 0.05$) during cheese ripening, either from evaporation or chemical or microbial degradation, whereas the concentration of 32 compounds did not vary significantly during ripening in any of the *P. fragi* cheeses. The remaining 49 volatile compounds, which increased significantly ($P < 0.05$) during cheese ripening, were produced by at least one of the five *P. fragi* strains. The higher production of esters, hydrocarbons, sulfur compounds, and aromatic compounds by *P. fragi* strains in the outer part of cheeses than in the inner part (Table 2) was in accordance with both their higher counts and aerobic metabolism.

The pattern of production of volatile compounds during ripening differed among strains. Esters were by far the most abundant group of volatile compounds in *P. fragi* cheeses, in contrast with the volatile profiles of cheeses made from milk inoculated with *Enterobacteriaceae* or *L. lactis* strains *(18, 19)*. Many of the esters detected in our *P. fragi* cheeses were present in cold stored milk *(28)* and in cheeses made from refrigerated raw ewes' milk *(1, 2)* and have been identified as odor-active compounds in different cheese varieties *(6)*. Production of esters by *Pseudomonas* spp. in milk has been associated with the occurrence of fruity aromas *(3, 11, 16, 21)*. Thus, ethyl esters of butanoic, hexanoic, and 3-methyl butanoic acids were related to the strawberry-like odor caused by some *P. fragi* strains. Previous reports *(3, 13, 23)* have highlighted the role of *P. fragi* strains as producers of esters. However, according to the results obtained in this study, only *P. fragi* 21 and *P. fragi* 39 can be considered producers of ethyl esters. *P. fragi* is generally not regarded as an alcohol-producing bacterium, although it can produce small amounts of ethanol *(21)*. Ethanol might be a limiting factor for the production of ethyl esters. Nevertheless, ethanol levels in cheeses made from milk inoculated with *P. fragi* 4 and *P. fragi* 37, which did not produce ethyl esters, were at least similar to those found in cheeses made with strains producing ethyl esters. Low esterase activity seems to be therefore the limiting factor for the production of ethyl esters. Ethyl pentanoate, which has been reported as the major ester produced by *P. fragi (20)*, was a minor volatile compound in our cheeses, produced only by *P. fragi* 39. This contradiction can be explained by the different substrate conditions: Morin et al. *(20)* grew the *P. fragi* strains in a laboratory medium. In this work, ethyl ethanoate and ethyl hexanoate were the major ethyl esters. There is evidence that *P. fragi* can produce ethyl hexanoate from ethanol and tricaproin via alcoholysis *(14)*, and this pathway might also be relevant for the production of other esters.

With respect to non–ethyl esters, *P. fragi* 21 and *P. fragi* 39 were the strains producing the highest amounts of these volatile compounds, followed by *P. fragi* 26. Production of non–ethyl esters by the latter strain, which did not produce ethyl esters, suggests the involvement of different mechanisms and enzymes in their synthesis, as shown for *P. fragi* CRDA 037 grown on whey *(9)*. Our results on the strain dependency of ester production by different *P. fragi* strains in cheese agree with those of Edwards et al. *(7)*, who found that ester production by *P. fragi* in beef stored at 6°C in air was strain dependent. However, our results contradict those of Urbach *(28)*, who reported ethyl ethanoate, ethyl butanoate, and ethyl hexanoate as the three major esters and methyl ethanoate as the only non–ethyl ester in the headspace of milk inoculated with *Pseudomonas* spp. strains held for 3 days at 7°C. Even though no acetic acid was detected, high levels of methyl ethanoate, ethyl ethanoate, and 2-propyl ethanoate were found in some of the *P. fragi* cheeses, pointing to a rapid exhaustion of acetic acid for ester formation.

Differences between *P. fragi* strains in the numbers and concentrations of alcohols produced in cheese were lower than for ester production. In four of the five *P. fragi* strains, the levels of total alcohols in the inner and outer parts of 12-day-old cheeses were very similar. Major alcohols in our cheeses were 2-propanol, ethanol, and 3-methyl butanol, a result almost coincident with the predominant alcohols in the headspace of refrigerated milk *(28)*. Ethanol, 3-methyl butanol, and 2-methyl propanol were the major alcohols in cheeses made from milk inoculated with *L. lactis* strains and in cheeses made from milk inoculated with *Enterobacteriaceae* strains *(18, 19)*. The presence of these two branched-chain primary alcohols, but not of the respective aldehydes, in our cheeses indicates a rapid reduction of 3-methyl butanal and 2-methyl propanal formed from leucine and valine. A strong decrease in 2-methyl propanal levels from day 1 to day 8 in cheeses made from milk inoculated with *Enterobacteriaceae* strains, with a concomitant increase in the levels of 2-methyl propanol, was reported *(18)*.

Undecene was the major hydrocarbon in our cheeses, although it was found only in the outer part, with *P. fragi* 26 as the highest producing strain. *P. fluorescens* and *P. putida* strains produced undecene on solid culture media *(15)*. However, this compound was absent from cheeses made from milk inoculated with strains of *L. lactis* or *Enterobacteriaceae (18, 19)*. Hydrocarbons, coming from lipid oxidation, have been reported frequently in the volatile fraction of cheeses *(26)*.

Dimethyl sulfide, dimethyl disulfide, and methyl thiocyanate were produced in significant amounts by the five *P. fragi* strains, with scarce differences in levels between strains. Sulfur compounds were not detected in cheeses made from milk inoculated with strains of lactococci, but dimethyl sulfide was present at high levels in cheeses made from milk inoculated with *Enterobacteriaceae* strains *(18)* and in the headspace of refrigerated milk *(28)*.

Ketones are a group of volatiles in which many odor-active compounds have been identified *(6)*. The low numbers and concentrations of ketones in our *P. fragi* cheeses agree with the absence of aldehydes, pointing to strong reducing environmental conditions. The five *P. fragi* strains produced 2-pentanone at similar levels in the inner and outer parts of 12-day-old cheeses, but acetoxypropanone was found only in cheeses with *P. fragi* 21. The two aromatic compounds found, styrene and *o*-dichlorobenzene, were produced by the five strains, and their levels were higher in the outer part than in the inner part of 12-day-old cheeses, in agreement with the higher counts and aerobic metabolism of *P. fragi*.

From the results obtained in this work, it can be concluded that *P. fragi* strains are capable of growth, survival, and production of a large variety of volatile compounds during cheese ripening. Even though most groups of volatile compounds were at higher levels in the outer part than in the edible inner part of cheeses and not all *P. fragi* strains were strong producers of volatile compounds, their presence might affect the sensory characteristics of cheese negatively. Lowering of *Pseudomonas* counts in milk, particularly when no heat treatment is applied before cheese manufacture, seems essential to prevent the appearance of undesirable volatile compounds.

ACKNOWLEDGMENTS

Financial support from INIA project OT02-004 and valuable help from Isabel Feliú in strain isolation and identification are acknowledged by the authors.

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