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Changes in the Volatile Composition of a Semihard Ewe Milk Cheese Induced by High-Pressure Treatment of 300 MPa

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The effect of high-pressure (HP) treatment (300 MPa, 10 min) on the volatile profile of semihard ewe milk cheeses was investigated. The HP treatment was applied at two different stages of ripening (1 and 15 days; 3P1 and 3P15) and microbiota, proteolysis indexes (soluble nitrogen and total free amino acid content), and volatile compounds were assayed at 15, 60, 90, and 150 days of ripening. The intensity of odor and aroma of cheeses was also assayed. 3P1 cheeses presented the highest content of free amino acids and were characterized by the lowest amounts of aldehydes, ketones, short-chain free fatty acids, and terpenes and higher levels of ethanol and ethyl esters. 3P15 cheeses were characterized by the highest content of short-chain free fatty acids and pyruvaldehyde and the lowest abundance of secondary alcohols and were more similar to control cheeses than those HP-treated on the first day. Intensities of odor and aroma were not significantly influenced by the HP treatment. However, the panellists found some differences in 3P1 as compared with control and 3P15 cheeses in what they perceived as lower odor and aroma quality.

KEYWORDS: High-pressure; volatile compounds; ewe milk cheese

INTRODUCTION

The volatile profile of cheeses is characteristic of each cheese variety and was derived from the glycolysis, lipolysis, proteolysis, and secondary catabolic reactions that take place throughout the ripening of cheeses. The ripening of hard and semihard cheeses is a long and costly process, and consequently, there are many methods available to accelerate it (*1*). The possible application of high hydrostatic (high-pressure, HP) technology for ripening acceleration has been studied by different authors (*2*) and requires the study of treatment conditions in each variety of cheeses. In a previous study, working with a range of pressures from 200 to 500 MPa, we tested the possibility of accelerating the proteolysis of ewe milk cheeses by HP treatment, concluding that 300 MPa was the optimum treatment for accelerating cheese ripening in this variety of cheeses (*3*).

Proteolysis involves the hydrolysis of caseins to peptides and free amino acids (FAAs), contributing directly to cheese flavor (*4*), and lipolysis releases short-chain free fatty acids (FFAs), which are important, or even predominant, components of the flavor of many types of ewe and goat milk cheeses (*5*). Subsequently, changes in the proteolysis and lipolysis rates due to HP treatment can affect the development of cheese flavor and therefore consumer acceptance.

Few works have investigated the effect of HP treatment on the volatile profile of cheeses. Butz et al. (*6*) found lower concentrations of *n*-butanoic acid and 3-hydroxy-2-butanone in pressurized Gouda cheese, whereas Saldo et al. (*7*) described lower amounts of FFA in pressurized goat milk cheeses. According to Jin and Harper (*8*), a treatment of 550 MPa for 30 min delayed the fermentation process and ripening and reduced volatile compound formation in Swiss cheese slurries. Ávila et al. (*9*) assayed the effect of HP treatment (400 MPa for 10 min) applied to 15 day old Hispánico cheeses and concluded that HP limited the formation of volatile compounds, decreasing the odor quality of cheeses.

The aim of the present work was to establish the effect of an HP treatment at 300 MPa and the possible influence of the ripening stage at which the treatment is applied (1 and 15 days) on the headspace volatile profile of ewe milk cheeses.

MATERIALS AND METHODS

Cheese Making and HP Treatment. Two independent batches of cheeses were manufactured from pasteurized (75.5 °C, 1 min) ewe milk in a cheese factory (MontBru, Moia, Barcelona, Spain). Cheeses were produced by 1% of starter culture (*Lactococcus lactis* ssp. *lactis* and

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L. lactis ssp. *cremoris*, Sacco SRL, CO, Italia), 0.05% of $CaCl_2$ (w/v), 0.02% (v/v) of calf rennet (Renifor-10, 520 mg chymosin/L, Laboratorios Arroyo, Santander, Spain), and 0.01% of lysozyme. The coagulum was cut, drained, molded (14.5 cm × 8.5 cm), and pressed (45 min at 1.2 kPa, 45 min at 1.8 kPa, 45 min at 2.45 kPa, and 1 h at 3.1 kPa). Cheeses (~1.3 kg) were salted by immersion in brine (20% NaCl solution) for 4 h and ripened in a room at 12 °C and 85% relative humidity. One group of cheeses was treated at 300 MPa for 10 min in a batch isostatic press (GEC Alsthom ACB, Nantes, France) on the first day after cheese making (3P1), whereas another group was treated under the same conditions at 15 days of ripening (3P15). A third group of untreated cheeses, maintained in the ripening chamber, was used as a control.

Chemical Composition and Microbiology. Triplicate samples were assayed for total solids (*10*) and total nitrogen (*11*). The cheese nitrogen was fractioned according to the method of Kunchroo and Fox (*12*), and the extracts obtained were used to determine soluble nitrogen at pH 4.6 (water soluble nitrogen, WSN). Total FAAs were determined on the WSN by the cadmium−ninhydrin method described by Folkertsma and Fox (*13*). The pH was measured with a pH meter (Crison Micro-pH 2001) on a cheese/distilled water (1:1) slurry. Analyses were performed throughout ripening. Microbiological analyses of total counts, lactococci, and lactobacilli were performed as described by Juan et al. (*3*).

Analysis of Volatile Compounds. Cheeses were sampled for volatile compounds at 15, 60, 90, and 150 days of ripening. Cheeses were cut into sections, vacuum packed, and stored at −80 °C until the day before volatile analysis. Cheese sections were thawed at 4 °C overnight before volatile analysis.

SPME Procedure. Samples were prepared by removing 1 cm from the cheese surface in order to minimize the sampling of volatile compounds that could have migrated from the environment and then grated to a uniform grain size at 10−12 °C temperature. One gram of sample was taken and placed in a 4 mL vial, which was subsequently sealed with PTFE/silicone septa (Supelco, Bellefonte, PA). A 75 μm Carboxen-PDMS fiber (Supelco) in a manual SPME manual holder was used to concentrate the cheese headspace. Fiber was exposed to the headspace above the sample for 30 min at 80 °C and desorbed in the injection port for gas chromatography−mass spectrometry (GC-MS) analysis at 280 °C. We used high temperature (80 °C) to improve the extraction of volatiles compounds.

GC-MS Analysis. Headspace volatile compounds were analyzed using an 8000 series gas chromatograph acoupled to a MD 800 mass spectrometer detector (Fisons Instruments, Milan, Italy). Data were recorded and analyzed with the Xcalibur version 1.1 software (Thermo Finnigan, Manchester, United Kingdom). Analyses were performed using a supelcowax (Supelco) capillary column (60 m × 0.25 mm × 0.25 μm film thickness). The oven temperature was initially held at 40 °C for 10 min and then increased to 110 °C at a rate of 5 °C/min followed by 10 °C/min to 240 °C and finally held at 240 °C for 15 min. Helium was used as the carrier gas with a flow rate of 1 mL min^{-1}. The split valve was opened 5 min after injection. The transfer line from the gas chromatograph to the mass spectrometer was held at 250 °C. Chromatographic peaks were detected as described previously by Barron et al. (*5*). Peak identification was performed by comparing the mass spectra with the NBS (National Bureau of Standards, United States) and NIST (National Institute of Standards and Technology, United States) libraries and by comparison of their retention times with authentic standards when available. Peak areas (arbitrary units) were calculated from the total ion current.

Sensory Analysis. Fourteen panellists from the Centre Especial de Recerca Planta de Tecnologia dels Aliments (CERPTA) experienced in the sensory assessment of cheese participated in the sensory analysis. They tasted the cheeses at 30 and 90 days of ripening to evaluate the intensity of odor and aroma of cheeses. Prior to assessment, the outer rind of each cheese was removed and cheeses were cut in triangular samples. The odor was described as an organoleptic property perceived by the olfactory organ when the cheese was smelled, and the aroma was described as the organoleptic property perceived by the olfactory organ through the retronasal passage when the cheese was tasted in the mouth. Each attribute was evaluated on a seven-point intensity scale anchored with "null or very weak" on the left end (*1*) and "high" on the right end of the scale (*7*).

Statistical Analysis. Multifactor analysis of variance was performed on data from two batches of cheeses for composition, microbiology, proteolysis, volatile compounds, and sensory analysis by SPSS Win version 12.0 (SPSS Inc., Michigan), using HP treatment and ripening time as the main factors. On the other hand, means for the different treatments (control, 3P1, and 3P15) at 15, 60, 90, and 150 days of ripening were compared using Student−Newman−Keuls test. Evaluations were based on a significance level of $P \leq 0.05$. Principal component analysis (PCA) was performed using Statistica^R Software (6.0 version, Statsoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Composition and Microbiology. Cheeses presented 57.5 ± 2.43% of fat and 34.5 ± 1.52% of protein, both expressed as % of the dry matter basis. The moisture content decreased during ripening in all cheeses (**Table 1**). At the beginning, HP-treated cheeses presented lower moisture content than control cheeses, due to water expulsion caused by the HP treatment applied. However, during ripening, HP-treated cheeses had better water retention properties than control cheeses, with 3P1 cheeses showing the highest moisture content at 90 days of ripening. This could be explained by the fact that HP treatment causes changes in the cheese protein network, forming a new structure that better retains the water in cheeses. No significant differences in moisture content were found between cheeses at 5 months old. 3P1 Cheeses showed the highest level of FAA on all ripened dates sampled (**Table 1**), agreeing with a previous study (*3*). The changes produced in the casein network by HP could improve the enzyme−substrate interaction promoting the formation of FAA in 3P1 cheeses. On the other hand, although differences were not statistically significant, 3P15 cheeses had higher levels of WSN as compared to 3P1 and control cheeses. Overall, 3P1 cheeses presented the highest pH levels (**Table 1**) during ripening, differences that could be explained by the decrease in the starter counts responsible for lactose fermentation and the production of lactic acid. 3P15 Cheeses presented similar pH values to control cheeses, indicating that the acidification process was completed at the initial stages of ripening.

Pressure treatment on the first day of ripening caused a decrease of 2 and 3 log units in lactococci and lactobacilli, respectively (**Table 2**). Both were recovered during the ripening time. Treatment at 15 days of ripening caused a lower decrease in bacteria counts (2 log units in lactococci and 1 log unit in lactobacilli), but their recovery was slower than in P1 cheeses. At 60 days of ripening, no differences in microbial counts were found between cheeses.

Volatile Compounds. Fifty-five compounds were identified in the headspace of the control and HP-treated ewe milk cheeses. **Tables 3−8** show the abundance (integrated area counts) of acids, ketones, aldehydes, alcohols, ethyl esters, lactones, and terpenes after 15, 60, 90, and 150 days of ripening. Most of them have been previously detected in some ewe milk cheese varieties (*5*, *14*−*16*). Eleven volatile compounds (*n*-hexanoic acid, 2-decanone, phenylmetanal, phenylethanal, 2,3-dimethyl-pentanal, 3-methyl-butanal, 3-methyl-butanol, 2,3-butanediol, δ-hexalactone, γ-hexalactone, and δ-decanolactone) were not significantly influenced ($P > 0.05$) by the HP treatment.

Acids. Five acids were detected in our cheeses (**Table 3**). As the ripening time increased, the abundance of acids increased due to lipolysis process. At 90 days of ripening, acids slightly decreased their abundance; however, the level of each individual acid at the end of ripening was markedly increased as compared to those at 15 days. *n*-Hexanoic and *n*-heptanoic acids did not

Table 1. Composition and Proteolysis of Control and HP-Treated Ewe Milk Cheeses during Ripening[a]

	days of ripening														effects (P)[b]		
	15			60			90			150							
	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	R	T	R × T
pH	4.92 ± 0.04 c	5.25 ± 0.01 a	4.95 ± 0.04 b	4.8 ± 0.72 c	4.95± 0.11 a	4.91 ± 0.05 b	4.74 ± 0.05 b	4.92 ± 0.05 a	4.74 ± 0.08 b	4.9 ± 0.09 a	4.91 ± 0.05 a	4.86 ± 0.05 b	***	***	***
moisture (%)	42.35 ± 0.20 a	40.33 ± 0.30 b	39.85 ± 0.12 c	35.46 ± 0.60 a	35.34 ± 0.18 a	33.37 ± 0.11 b	29.46 ± 0.12 c	31.69 ± 0.17 a	30.12 ± 0.54 b	24.46 ± 0.56	24.69 ± 0.67	24.79 ± 0.78	***	***	***
FAA (mg Leu/g)	0.62 ± 0.14 b	1.53 ± 0.11 a	0.61 ± 0.14 b	2.18 ± 0.12 c	4.80 ± 0.19 a	3.48 ± 0.10 b	4.85 ± 0.15 b	7.71 ± 0.32 a	5.49 ± 0.12 b	7.73 ± 0.73 c	14.97 ± 3.27 a	11.13 ± 0.54 b	***	***	NS
WSN/TN (%)	12.19 ± 1.16	11.02 ± 0.44	15.30 ± 2.91	19.39 ± 0.57	18.77 ± 1.46	19.99 ± 0.89	22.15 ± 0.33 a	19.03 ± 0.27 b	22.98 ± 0.67 a	24.75 ± 1.6	23.79 ± 1.75	25.64 ± 1.79	***	**	NS

[a] Means ± SD for the same parameter and day of ripening by different letters are significantly ($P \leq 0.05$) different. Control, untreated cheeses; 3P1, cheeses treated at 300 MPa on the first day of manufacturing; and 3P15, cheeses treated at 300 MPa at 15 days of manufacturing. [b] Statistical significance: T, HP treatment; R, ripening time; ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.1$; and NS, not significant.

appear until 60 and 150 days of ripening, respectively. Lipolysis is particularly extensive in hard Italian cheese varieties, surface bacterially ripened cheeses, and blue mold cheeses (*17*). Our cheeses presented a moderate lipolysis, essentially due to the heat treatment applied to cheese milk (75.5 °C, 1 min), which inactived indigenous milk lipase, and to the weak lipolytic activity associated with lactic acid bacteria used as starter (*18*).

The contribution of FFA to aroma development has been demonstrated in many mature cheese varieties (*7*). Furthermore, fatty acids are not only aroma compounds by themselves but also serve as precursors of methyl ketones, alcohols, lactones, and esters.

Ethanoic acid showed the highest area value in each sample at all ripening stages; *n*-butanoic, *n*-pentanoic, *n*-hexanoic, and *n*-heptanoic acids followed in decreasing order (**Table 3**). Ethanoic acid, with a characteristic strong sharp-vinegar note, can originate from a number of sources, including the oxidation of lactose by starter bacteria under anaerobic conditions (*19*), the oxidation of lactate by nonstarter lactic acid bacteria (NSLAB) (*20*), and the catabolism of amino acids (such as alanine and serine) by NSLAB and starter bacteria (*21*).

At 15 days of ripening, the abundance of the total acids in HP-treated cheeses was lower than control cheeses, but afterward, these compounds tended to rise, and although the highest levels were found in 3P15 cheeses, differences were not statistically significant at 60, 90, and 150 days of ripening (**Table 3**). The lower abundance of acids found in the HP-treated cheeses at 15 days of ripening may be due to the decrease of lactic acid bacteria counts produced by the pressure. These results agreed with those of Saldo et al. (*7*) who found lower amounts of fatty acids in HP-treated Garrotxa cheeses (400 MPa) as compared to untreated cheeses. These authors suggested that pressure might have inactivated some lipases from secondary microbiota in cheese, decreasing the lipolysis and subsequent formation of FFA. The microbiota of our cheeses recovered with time. In fact, total bacteria counts of 3P1 and 3P15 cheeses did not differ from untreated cheeses at 30 and 60 days of ripening, respectively (**Table 2**). Moreover, it is important to consider that the location of lipase activities appears to be intracellular and requires release into the cheese matrix through cell lysis for maximum efficiency (*18*). Lortal and Chapot-Chartier (*22*) suggested a positive link between lysis of lactic acid bacteria and lipolysis. In a previous study, it was observed that pressures of 300 MPa could cause a cell lysis (*3*), which could improve the release of lipase to the cheese matrix. At 15 days of ripening, the levels of lactobacilli in cheeses were higher than those on the first day. As a consequence, when cheeses were submitted to HP treatment, higher levels of lipolytic enzyme could be released to the cheese matrix in 3P15 cheeses than in 3P1 cheeses, which could explain the higher abundance of acids found in 3P15 cheeses at 60 and 90 days of ripening (**Table 3**).

Ketones. Methyl ketones are intermediate compounds formed by oxidation of FFAs to β-ketoacids and decarboxylation to methyl ketones with one less carbon atom (*17*). Abundances of the total methyl ketones increased until 90 days of ripening, decreasing thereafter (**Table 4**). The conversion of methyl ketones into the corresponding secondary alcohols could be responsible for the variations in the abundances of methyl ketones during ripening (*23*).

2-Pentanone, 2-heptanone, and 2-nonanone were the main 2-methyl ketones, whereas 2-butanone, 2-octanone, and 2-decanone were the minor ketones throughout the ripening (**Table 4**). At 15 days of ripening, the abundance of ketones in HP-treated cheeses was lower than control cheeses, but afterward,

Table 2. Microbiological Analysis (log cfu g^{-1}) of Control and HP-Treated Ewe Milk Cheeses during Ripening[a]

microbial counts (log cfu g^{-1})	days of ripening														effects (P)[b]		
	1		15			30			60			90					
	control	3P1	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	R	T	R × T
lactococci	9.25 a	6.93 b	9.28 a	8.19 ab	7.11 b	8.2 a	8.4 a	7.04 b	7.89	8.33	8.08	7.6	7.67	7.76	NS	***	***
lactobacilli	6.54 a	2.83 b	7.58 a	7.88 a	6.63 b	7.7	7.81	6.61	8.07	8.42	8.06	8.02	7.88	7.95	***	***	***
total counts	9.22 a	7.82 b	9.14 a	8.36 ab	7.32 b	8.23 a	8.59 a	7.13 b	8.14	8.61	8.23	7.9	7.98	7.95	NS	**	*

[a] Means for the same row and day of ripening by different letters are significantly ($P \leq 0.05$) different. Control, untreated cheeses; 3P1, cheeses treated at 300 MPa on the first day of manufacturing; and 3P15, cheeses treated at 300 MPa at 15 days of manufacturing. [b] Statistical significance: T, HP treatment; R, ripening time; ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.1$; and NS, not significant.

Table 3. Abundance[a] of Acids Detected in the Headspace of Volatile Fraction in Ewe Milk Cheeses[b]

			days of ripening												effects (P)[e]		
			15			60			90			150					
	RT[c]	ID[d]	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	R	T	R × T
ethanoic	30.01	MS(P)	2586	1510	1798	3091	2140	3872	3250	1333	4294	4932b	3513b	7371a	***	***	*
butanoic	33.06	MS(P)	314	234	141	1617 b	1340 c	1881 a	1015 b	1304 b	1866 a	2298 b	2988 a	1850 b	***	*	***
pentanoic	36.49	MS(P)	77	56	55	489 b	396 b	730 a	497 b	538 b	940 a	878	1112	878	***	**	***
hexanoic	39.16	MS(P)	ND	ND	ND	62	49	54	86	91	95	150	308	154	***	NS	*
heptanoic	42.16	MS(P)	ND	ND	ND	ND	ND	ND	ND	ND	ND	16b	70 a	21 b	***	***	***
total		MS(P)	2977 a	1800 b	1994 b	5260	3925	6536	4848	3266	7195	8274	7992	10275	***	***	*

[a] Integrated area counts; ND, not detectable levels. [b] Means for the same volatile compound and day of ripening by different letters are significantly ($P \leq 0.05$) different. Control, untreated cheeses; 3P1, cheeses treated at 300 MPa on the first day of manufacturing; and 3P15, cheeses treated at 300 MPa at 15 days of manufacturing. [c] Retention time (min). [d] Identification: MS, mass spectra; (T), tentatively identified on the basis of the NBS and NIST libraries; (P), positively identified by comparison with RT and MS of authentic standards. All components were quantified with total ion chromatogram (TIC). [e] Statistical significance: T, HP treatment; R, ripening time; ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.1$; and NS, not significant.

Table 4. Abundance[a] of Ketones Detected in the Headspace of Volatile Fraction in Ewe Milk Cheeses[b]

			days of ripening												effects (P)[e]		
			15			60			90			150					
	RT[c]	ID[d]	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	R	T	R × T
2-propanone	5.23	MS(T)	91	80	68	228	50	82	122	39	115	370 a	49 ab	224 b	***	***	**
2-butanone	7.29	MS(P)	18	5	7	20	ND	13	25	ND	9	29	9	18	*	***	NS
2-pentanone	10.63	MS(P)	163	177	101	1659	269	787	471	295	817	1308 a	409b	1314 a	***	***	*
2-heptanone	20.73	MS(T)	396 a	232 b	82 c	1805 a	582 b	1467 a	2524 a	1078 b	1334 b	1931 a	790 b	1197 b	***	***	***
2-octanone	24.76	MS(P)	ND	ND	ND	13	ND	14	27	ND	14	15 a	ND	8 b	***	***	***
2-nonanone	27.86	MS(T)	205 a	111a b	12 b	622 b	296 c	818 a	1869	1678	1404	436	427	446	***	**	***
2-decanone	32.29	MS(P)	ND	ND	ND	ND	ND	ND	25	22	21	10	12	16	***	NS	NS
8-nonen-2-one	29.23	MS(T)	ND	ND	ND	19a	11b	24a	54	34	41	23	13	13	***	**	*
2.3-pentanedione	15.19	MS(T)	ND	ND	ND	ND	ND	ND	36 a	ND	23 b	ND	ND	ND			
2.3-butanedione	10.89	MS(T)	292 a	117 b	242 ab	146	43	420	71 b	ND	95 a	79 b	17 b	313 a	***	***	***
total			1164 a	721 b	511 b	4504 a	1150 b	3625 a	5239	3164	3882	4200 a	1726 b	3555 a	***	***	**

[a] Integrated area counts; ND, not detectable levels. [b] Means for the same volatile compound and day of ripening by different letters are significantly ($P \leq 0.05$) different. Control, untreated cheeses; 3P1, cheeses treated at 300 MPa on the first day of manufacturing; and 3P15, cheeses treated at 300 MPa at 15 days of manufacturing. [c] Retention time (min). [d] Identification: MS, mass spectra; (T), tentatively identified on the basis of the NBS and NIST libraries; (P), positively identified by comparison with RT and MS of authentic standards. All components were quantified with total ion chromatogram (TIC). [e] Statistical significance: T, HP treatment; R, ripening time; ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.1$; and NS, not significant.

these compounds tended to rise in 3P15 cheeses, reaching similar values to the untreated ones. On the other hand, 3P1 cheeses presented the lowest methyl ketones levels at 60, 90, and 150 days of ripening (**Table 4**).

Formation of these compounds is mainly a result of the lipolytic action of the microbiota in the cheeses. At day 15 of ripening, HP cheeses presented lower bacteria counts than control cheeses (**Table 2**); as a consequence, lower abundance of FFA and methyl ketones was found in HP-treated cheeses. The presence of 2,3-butanedione (diacetyl) and 2-butanone was a consequence of the lactose and citrate metabolisms by starter bacteria and NSLAB, respectively (*24*).

At 15 days of ripening, HP-treated cheeses presented lower amounts of 2,3-butanedione than control cheeses. HP treatment decreased counts of lactic acid bacteria (**Table 2**); as a result, a diminution of lactose fermentation, with a subsequent reduction of 2,3-butanedione, was observed in HP-treated cheeses. The treatment applied (300 MPa) allowed a recovery of lactic bacteria, which reached similar counts to untreated cheeses at 60 days of ripening (**Table 2**). At this moment, the highest and lowest abundances of 2,3-butanedione were found in 3P15 and 3P1 cheeses, respectively. 2,3-Butanedione could be reduced to 3-hydroxy-2-butanone by starter bacteria and then be converted to 2,3-butanediol and 2-butanone by adventitious bacteria (*24*) or chemical reduction (*25*). 2-Butanone increased during ripening, and the lowest levels were found in 3P1 cheeses, in accordance with the lowest level of 2,3-butanedione present in these cheeses (**Table 4**).

Table 5. Abundance[a] of Aldehydes Detected in the Headspace of Volatile Fraction in Ewe Milk Cheeses[b]

			days of ripening														
			15			60			90			150			effects (P)[e]		
	RT[c]	ID[d]	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	R	T	R × T
ethanal	3.96	MS(P)	588 a	227 b	281 b	205	134	148	110	90	120	122	104	121	***	***	***
hexanal	16.13	MS(T)	164 a	65 b	84 b	541	275	397	359	72	290	ND	ND	ND	***	***	*
heptanal	20.86	MS(T)	ND	ND	ND	ND	94	ND	ND	ND	ND	ND	ND	ND			
octanal	24.86	MS(T)	10 a	ND b	ND b	16 a	10 b	18 a	22 a	ND b	18 a	ND	ND	ND	***	***	***
nonanal	27.99	MS(T)	80 a	35 b	10 b	116	54	132	114	107	122	37	36	37	**	NS	NS
phenylmethanal	31.19	MS(T)	26	2	11	56	62	6	61	30	35	41	51	46	***	NS	*
phenylethanal	33.29	MS(T)	ND	ND	ND	ND	ND	ND	63	80	86	61	107	62	***	NS	NS
branched chain aldehydes																	
2.3-dimethyl-pentanal	4.83	MS(T)	ND	ND	ND	27	14	21	17	ND	19	ND	ND	ND	***	*	NS
3-methyl-butanal	7.83	MS(T)	19	19	16	44	55	41	32	25	28	45	42	41	***	NS	NS
pyruvaldehyde	25.19	MS(T)	1478	186	1419	96	ND	1344	ND	ND	64	ND b	ND b	345 a	***	***	***
total[f]			886 a	347 b	400 b	1005 a	603 b	822 ab	779 a	394 b	717 a	306	339	307	***	***	***
total			2364 a	533 b	1819 a	1101 b	603 c	2166 a	779 a	404 b	781 a	306 b	339 b	652 a	***	***	***

[a] Integrated area counts; ND, not detectable levels. [b] Means for the same volatile compound and day of ripening by different letters are significantly ($P \leq 0.05$) different. Control, untreated cheeses; 3P1, cheeses treated at 300 MPa on the first day of manufacturing; and 3P15, cheeses treated at 300 MPa at 15 days of manufacturing. [c] Retention time (min). [d] Identification: MS, mass spectra; (T), tentatively identified on the basis of the NBS and NIST libraries; (P), positively identified by comparison with RT and MS of authentic standards. All components were quantified with total ion chromatogram (TIC). [e] Statistical significance: T, HP treatment; R, ripening time; ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.1$; and NS, not significant. [f] Total aldehydes without pyruvaldehyde.

Aldehydes. Aldehydes do not accumulate in cheese because they are rapidly reduced to primary alcohols or even oxidized to the corresponding acids (*26*). At 15 days of ripening, the abundance of aldehydes in HP-treated cheeses was lower than control cheeses, but afterward, these compounds tended to rise in 3P15 cheeses, with 3P1 cheeses showing the lowest levels at 60 and 90 days of ripening, and, except for pyruvaldehyde, no significant differences were observed between cheeses at the end of ripening (**Table 5**).

Ethanal is the most common aldehyde found in fermented dairy products and is derived from lactose fermentation (*27*) and breakdown of threonine (*28*). HP treatment reduced counts of lactic bacteria (**Table 2**); thus, a reduction of lactose fermentation could then occur in HP cheeses, which would explain the lower abundance of ethanal observed in HP cheeses at 15 days of ripening. Straight-chain aldehydes, such as *n*-hexanal, *n*-heptanal, *n*-octanal, and *n*-nonanal, may be formed from β-oxidation of unsaturated fatty acids and are characterized by green-grass and herbaceous aromas. At 15 days of ripening, a lower abundance of FFAs was detected in HP cheeses as compared to untreated ones, a fact that might explain the lower *n*-hexanal, *n*-octanal, and *n*-nonanal levels observed in HP cheeses. Levels of pyruvaldehyde decreased significantly in 3P1 cheeses but increased in 3P15 cheeses. Pyruvaldehyde is produced through glicolysis by lactic acid bacteria and is involved in the production of cheese flavor. At 15 days of ripening, 3P1 cheeses presented the lowest amounts of pyruvaldehyde probably due to a decrease in lactic bacteria caused by HP. Pyruvaldehyde quickly reacts with amino compounds producing several volatile components (*29*); however, it seems that the treatment applied at 15 days of ripening significantly affected the normal behavior of this compound, obtaining the highest levels in 3P15 cheeses at 60, 90, and 150 days of ripening.

3-Methyl-butanal and phenylethanal were formed from leucine and phenylalanine, respectively. The phenylmethanal biosyntesis pathway is not very well-known but could originate from an α-oxidation of phenylethanal or from a β-oxidation of cinnamic acid (*30*). It can also be produced by a reaction of pyruvaldehyde with phenylalanine (*31*). In our cheeses, levels of 3-methyl-butanal, phenylmethanal, and phenylethanal were unaffected by HP treatment (**Table 6**).

Alcohols. The abundance of primary and secondary alcohols was influenced by HP treatment (**Table 6**). In general, HP decreased the alcohol levels in 3P15 cheeses and increased levels of ethanol in 3P1 cheeses. Ethanol can be derived from lactose fermentation by starter bacteria (*32*), amino acid catabolism (Strecker degradation of alanine), or from ethanal reduction (*30*). Furthermore, ethanol is the precursor of ethyl esters, volatile compounds relevant to cheese aroma (*30*). Pressures of 300 MPa on the first day of ripening produced an increase in the levels of amino acids (**Table 1**), which are one of the precursors of ethanol by Strecker degradation and could explain the highest levels of ethanol found in 3P1 cheeses in all ripening stages.

Primary and secondary alcohols are produced by the reduction of their corresponding aldehydes and ketones, respectively (*30*). At 15 days of ripening, HP-treated cheeses had lower abundances of alcohols (except ethanol) than untreated cheeses (**Table 6**), which could be explained by the lower levels of aldehydes and ketones found in HP-treated cheeses as compared to the controls (**Tables 4** and **5**). Lower amounts of 1-pentanol and 2-pentanol were found in HP-treated cheeses throughout ripening. However, 3P1 cheeses presented the highest levels of 1-butanol and 1-hexanol at 60 days of ripening and 2-nonanol at 90 and 150 days of ripening. The lowest contents of secondary alcohols were found in 3P15 cheeses, being undetectable at 60 and 150 days of ripening. A very low abundance of 3-methyl-butanol was found in cheeses at 15 days of ripening, and no significant differences were found between HP-treated and control cheeses (**Table 6**).

2,3-Butanediol, generated by reductions of 2,3-butanedione and 3-hydroxy-2-butanone, was also only observed at 15 days of ripening, surely due to its further reduction to 2-butanone by nonstarter bacteria (*33*). The abundance of both compounds showed no statistically significant differences between cheeses.

Esters. Esters are common constituents of cheese volatiles. Most of them are described as having fruity, floral notes, and some of these esters have a very low perception threshold. In our cheeses, only ethyl esters were detected, which are primarily formed by esterification between short- to medium-chain fatty acids and ethanol (**Table 7**).

The abundance of ethyl esters was significantly affected by HP treatment. At 15 days of ripening, control cheeses presented the highest abundance of ethyl esters, corresponding to their

Table 6. Abundance[a] of Alcohols Detected in the Headspace of Volatile Fraction in Ewe Milk Cheeses[b]

			days of ripening												effects (P)[e]		
			15			60			90			150					
	RT[c]	ID[d]	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	R	T	R × T
primary alcohols																	
ethanol	8.59	MS(P)	265 b	2763 a	283 b	410 b	5991 a	187 b	417 b	4549 a	519 b	76 b	4828 a	332 b	**	***	**
1-butanol	19.26	MS(P)	ND	ND	ND	ND b	47 a	ND b	ND	ND	ND	ND	ND	ND			
1-pentanol	23.56	MS(P)	15 a	7 b	4 b	13 a	ND b	ND b	ND	ND	ND	ND	ND	ND	***	***	***
1-hexanol	26.86	MS(P)	7	13	13	6 b	37 a	10 b	ND	ND	ND	ND	ND	ND	***	***	***
secondary alcohols																	
2-pentanol	18.46	MS(T)	32 a	9 b	8 b	221	208	ND	175 a	144 b	46c	302 a	160 b	ND c	***	***	***
2-heptanol	25.86	MS(P)	13 a	7 b	ND c	62 b	181 a	ND b	129 a	159 a	ND b	197 a	107 b	ND c	***	***	***
2-nonanol	30.66	MS(P)	ND	ND	ND	ND	ND	ND	33 ab	37 a	23 b	ND b	13 a	ND b	***	***	***
branched chain alcohols																	
3-methyl-butanol	21.93	MS(T)	6	10	4	ND	ND	ND	ND	ND	ND	ND	ND	ND	***	***	***
2.3-butanediol	32.46	MS(T)	331	352	298	ND	ND	ND	ND	ND	ND	ND	ND	ND	***	NS	NS
total			669 b	3161 a	606 b	711 b	6301 a	197 b	771 b	4903 a	587 b	575 b	5107 a	332 b	*	***	**

[a] Integrated area counts; ND, not detectable levels. [b] Means for the same volatile compound and day of ripening by different letters are significantly ($P \leq 0.05$) different. Control, untreated cheeses; 3P1, cheeses treated at 300 MPa on the first day of manufacturing; and 3P15, cheeses treated at 300 MPa at 15 days of manufacturing. [c] Retention time (min). [d] Identification: MS, mass spectra; (T), tentatively identified on the basis of the NBS and NIST libraries; (P), positively identified by comparison with RT and MS of authentic standards. All components were quantified with total ion chromatogram (TIC). [e] Statistical significance: T, HP treatment; R, ripening time; ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.1$; and NS, not significant.

Table 7. Abundance[a] of Ethyl Esters Detected in the Headspace of Volatile Fraction in Ewe Milk Cheeses[b]

			days of ripening												effects (P)[e]		
			15			60			90			150					
	RT[c]	ID[d]	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	R	T	R × T
ethyl ethanoate	6.83	MS(T)	11	29	12	73 b	688 a	42 b	107 b	553 a	100 b	25 b	225 a	19 b	**	***	*
ethyl butanoate	13.86	MS(T)	244 a	73 b	33 b	209 b	1213 a	123 b	310	620	207	188 b	521 a	100 b	*	**	*
ethyl hexanoate	22.76	MS(P)	ND	ND	ND	120 b	765 a	109 b	342 b	2337 a	286 b	33 b	462 a	13 b	***	***	***
ethyl heptanoate	26.23	MS(T)	ND	ND	ND	6 b	24 a	18a b	ND	19	ND	ND	ND	ND	***	***	***
ethyl octanoate	28.89	MS(P)	ND	ND	ND	10	149	ND	64 b	111 a	65 b	11 b	304 a	10 b	***	***	***
ethyl decanoate	32.86	MS(P)	ND	ND	ND	ND	239 a	ND	18 b	452 a	21 b	ND	600 a	17 b	***	***	***
ethyl undecanoate	35.83	MS(T)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	13	ND			
ethyl dodecanoate	35.93	MS(T)	ND	ND	ND	ND	ND	ND	ND	17	ND	11 b	46 a	11 b	***	***	***
ethyl tetradecanoate	38.53	MS(T)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	20	ND	***	***	***
total			256 a	102 b	44b	418 b	3078 a	292 b	840 b	4110 a	679 b	268 b	2191 a	171 b	***	***	***

[a] Integrated area counts; ND, not detectable levels. [b] Means for the same volatile compound and day of ripening by different letters are significantly ($P \leq 0.05$) different. Control, untreated cheeses; 3P1, cheeses treated at 300 MPa on the first day of manufacturing; and 3P15, cheeses treated at 300 MPa at 15 days of manufacturing. [c] Retention time (min). [d] Identification: MS, mass spectra; (T), tentatively identified on the basis of the NBS and NIST libraries; (P), positively identified by comparison with RT and MS of authentic standards. All components were quantified with total ion chromatogram (TIC). [e] Statistical significance: T, HP treatment; R, ripening time; ***$P \leq 0.001$; **$P \leq 0.01$; and *$P \leq 0.1$.

higher levels of FFAs (**Table 3**). However, as the ripening time advanced, 3P1 cheeses presented the highest abundance of ethyl esters (**Table 7**), which in turn exhibited the highest abundance of ethanol available for esterification (**Table 6**).

As the ripening time increased, new ethyl esters appeared; ethyl decanoate, ethyl dodecanoate, and ethyl undecanoate and tetradecanoate were found at 60, 90, and 150 days, respectively. All of them first appeared in 3P1 cheeses; however, during ripening, they also appeared in the rest of the cheeses.

Lactones. Lactones are cyclic compounds formed by the intramolecular esterifications of hydroxy fatty acids (*17*). Ur-Rehman et al. (*34*) suggested that indigenous enzymes and NSLAB played some role in the formation of lactones, but the actions of microorganisms on lactone production in cheeses have never been clearly elucidated (*30*). Levels of lactones were unaffected by the HP treatment (**Table 8**) in agreement with Saldo et al. (*7*) who reported similar concentrations in goat milk cheeses submitted to 400 MPa for 5 min as compared to untreated cheeses.

Miscellaneous Compounds. Two terpens (limonene and phellandrene) and other hidrocarbons [hexane, toluene, xylene, ethylbenzene, 1-methyl-2(methylethyl)benzene, acetophenone, and styrene] were also found in cheeses (data not shown). Terpens presumably come from animal feed (*14*) and, therefore, are important for determining the geographical origin of a cheese type, but their importance in the formation of cheese flavor remains uncertain (*26*). Xylene may presumably have a feed plant origin, and benzyl compounds can derive from the degradation of caroten in milk. Only a few levels of styrene were detected in untreated cheeses at 90 days of ripening. The HP treatment did not have a clear effect on these compounds.

Sensory Analysis. Odor and aroma intensities were not significantly influenced by the HP treatment (data not shown). However, panellists found some differences between cheeses in what they perceived as quality. 3P1 cheeses were described as lower odor and aroma qualities as compared to control and 3P15 cheeses.

Principal Components Analysis. PCA was applied to FAA, WSN, moisture, pH, and groups of volatile compounds according to the chemical nature. Ethanol and pyruvaldehyde were treated individually because their behavior differed significantly from their corresponding chemical groups.

Table 8. Abundance[a] of Lactones and Terpenes Detected in the Headspace of Volatile Fraction in Ewe Milk Cheeses[b]

			days of ripening												effects (P)[e]		
			15			60			90			150					
	RT[c]	ID[d]	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	R	T	R × T
								lactones									
γ-hexalactone	34.39	MS(P)	ND	ND	ND	13	7	11	17	14	42	20	16	76	*	NS	NS
δ-hexalactone	35.76	MS(T)	5	4	6	ND	5	ND	ND	ND	ND	ND	ND	ND	***	***	***
δ-decanolactone	41.16	MS(P)	ND	ND	ND	ND	ND	ND	ND	ND	ND	17	24	16	***	NS	*
total			5	4	6	13	12	11	ND	ND	ND	37	40	92	**	NS	NS
								terpenes									
limonene	20.99	MS(T)	ND	ND	ND	185 a	32c	91b	200	119	165	549 b	176 c	774 a	***	***	***
phellandrene	22.86	MS(T)	9 a	ND b	ND b	17 a	ND b	ND b	27 a	ND b	25a	138 a	35 b	184 a	***	***	***
total			9 a	ND b	ND b	202 a	32 c	91 b	227	119	191	687 b	211 c	958 a	***	***	***

[a] Integrated area counts; ND, not detectable levels. [b] Means for the same volatile compound and day of ripening by different letters are significantly ($P \leq 0.05$) different. Control, untreated cheeses; 3P1, cheeses treated at 300 MPa on the first day of manufacturing; and 3P15, cheeses treated at 300 MPa at 15 days of manufacturing. [c] Retention time (min). [d] Identification: MS, mass spectra; (T), tentatively identified on the basis of the NBS and NIST libraries; (P), positively identified by comparison with RT and MS of authentic standards. All components were quantified with total ion chromatogram (TIC). [e] Statistical significance: T, HP treatment; R, ripening time; ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.1$; and NS, not significant.

Table 9. Factor Scores (Means ± SD) from PC Analysis on Volatile Compounds, FAA, WSN, pH, and Moisture Content of Control, 3P1, and 3P15 Cheeses[a]

	days of ripening											
	15			60			90			150		
	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15	control	3P1	3P15
PC1	−1.39	−1.49	−1.22	−0.08 a	−0.50 b	−0.13 a	0.46 a	0.04 b	0.61 a	1.24 ab	0.58 b	1.62 a
PC2	0.95 a	−0.99 b	0.49 a	0.89 a	−1.37 b	1.22 a	0.58 a	−1.46 b	0.76 a	−0.31 b	−1.24 c	0.46 a

[a] Means for the same row and day of ripening by different letters are significantly ($P \leq 0.05$) different.

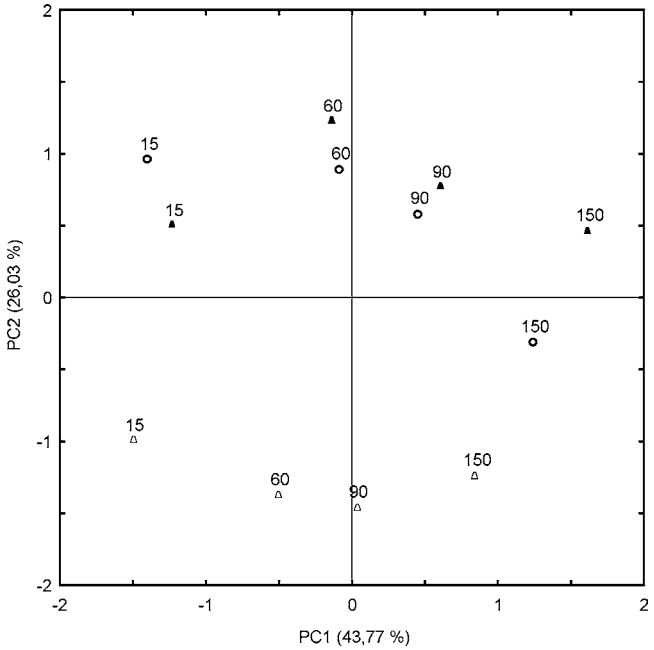

Figure 1. Plot depicting cheeses distribution (factor score mean values) from PC analysis of the volatile composition, FAAs, WSN, pH, and moisture content of control (○), 3P1 (△), and 3P15 cheeses (▲) at 15, 60, 90, and 150 days of ripening.

Table 9 and **Figure 1** shows the factor score mean values calculated for each cheese sample and time of ripening defined by principal components 1 (PC1) and 2 (PC2), which explained 43.77 and 26.03% of the variance, respectively. As observed, 3P1 cheeses highly differed from the other cheeses, whereas 3P15 cheeses were more similar to the control.

ABBREVIATIONS USED

HP, high-pressure; FAA, free amino acids; WSN, water-soluble nitrogen; FFA, free fatty acids; PCA, principal component analysis; NSLAB, nonstarter lactic acid bacteria.

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