Eur Food Res Technol (2014) 238:485–494
DOI 10.1007/s00217-013-2127-2

ORIGINAL PAPER

Volatile composition and improvement of the aroma of industrial Manchego cheese by using *Lactobacillus paracasei* subsp. *paracasei* as adjunct and other autochthonous strains as starters

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Received: 11 September 2013 / Revised: 17 October 2013 / Accepted: 4 November 2013 / Published online: 23 November 2013

Abstract The use of several autochthonous strains of lactic acid bacteria, including *Lactobacillus paracasei* subsp. *paracasei* as adjunct of the starter in the manufacture of Manchego cheese, was evaluated in an attempt to improve the aroma of the industrial Manchego cheese. Volatile composition and odour characteristics were evaluated and compared to those in Manchego cheese manufactured with a commercial starter (CS) culture and with raw milk cheese manufactured without starter. Manchego cheeses manufactured with two autochthonous strains of *Lactococcus lactis* subsp. *lactis* displayed a similar volatile profile and odour characteristics to the cheese made with the CS. The use of the strain *Lactobacillus paracasei* subsp. *paracasei* CECT 7882 as adjunct of the *Lactococcus* strains produced cheeses with higher amounts of some free fatty acids and alcohols, acetoin, lactones, phenylacetaldehyde, 2-phenylethanol and linalool, and higher scores of the odour intensity, odour quality, and ewe's milk odour than the CS cheeses. It resulted in an intensification and improvement of industrial Manchego cheese aroma.

Keywords Adjunct lactobacilli · Non-starter lactic acid bacteria · Manchego cheese · Volatile compounds · Sensory analysis

Introduction

Manchego cheese, the most popular Spanish cheese variety, is a cured, hard, and enzymatically coagulated cheese, made only in La Mancha region, with milk of Manchega breed ewes, and since 1985, it has been protected by a Denomination of Origin [1]. Manchego cheese can be artisanal or industrial, according to whether the cheeses are manufactured with raw or pasteurized milk. When pasteurized milk is used, spontaneous microbiota is partially destroyed and therefore milk is inoculated with commercial mixed-strain starter cultures, which generally comprise strains of *Lactococcus* (*Lc.) lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*.

It is well known that cheeses made with pasteurized milk ripen more slowly and lack full aroma of traditional cheeses made from raw milk (RM) [2], which has been related to the inactivation of indigenous milk enzymes and with the decrease in the number of non-starter lactic acid bacteria (NSLAB). Cheese flavour is a complex perception that results from the presence in different proportions of volatile and non-volatile compounds produced by enzymes

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from milk, rennet, and microorganisms [3]. Proteolytic enzymes from lactic acid bacteria are responsible for the degradation of casein and peptides leading to the production of free amino acids, which are then converted into specific volatile compounds by NSLAB and lactococci from the starter. In consequence, NSLAB together with starter LAB determine the organoleptic characteristics of the final cheese [4].

The NSLAB present during the ripening of artisanal Manchego cheese belong to the genera *Lactobacillus, Leuconostoc, Enterococcus,* and *Streptococcus* [5], being *Lactobacillus* the most abundant genus [6].

The survival of some NSLAB to the pasteurization and the post-pasteurization contamination introduce variability into the ripening process, representing one of the main factors that remains uncontrolled in industrial cheese-making [7]. Furthermore, the growth of some undesirable NSLAB can produce aroma defects in cheese during the ripening process. For these reasons, the dominance of appropriated NSLAB strains is crucial. The indirect control of the NSLAB microbiota by means of deliberately added adjunct cultures is considered the most suitable method [8].

Several research groups have evaluated the behaviour of different NSLAB strains, used alone or in combination with SLAB (starter lactic acid bacteria), in order to enrich the sensory characteristics of cheese [9] and to reproduce the flavour of RM cheeses when pasteurized milk is used [10, 11].

Preliminary studies from our research group in Manchego cheese [12–14] found that the addition of autochthonous strains of *Lc. lactis* and *Lactobacillus (Lb.) plantarum* as an adjunct produced significantly higher amounts of free amino acids, a more complex volatile profile and higher sensory scores than those obtained using a commercial starter (CS) culture. In these previous studies, strains used for cheese-making had not been genetically and technologically characterized.

More recent studies carried out at our laboratory [15–17] from good-quality RM Manchego cheeses have allowed the isolation, genetic characterization, and the selection of some autochthonous lactic acid bacteria strains, on the basis of their prevalence during the cheese ripening, and safety and technological parameters such as acidifying, autolytic and proteolytic activities. These strains were proposed to be used as starters or adjunct cultures in the manufacture of pasteurized milk Manchego cheeses. The aim of this work was to evaluate the effect of the use of these strains on the volatile composition and to improve the odour sensory characteristics of industrial Manchego cheese.

Materials and methods

Starter cultures for cheese-making

Lc. lactis subsp. *lactis* CECT 7883 (B8W3), *Lc. lactis* subsp. *lactis* CECT 7884 (A0W2) and *Lb. paracasei* subsp. *paracasei* CECT 7882 (PBL226) were used. These were obtained from the culture collection of the Food Science and Technology Department of the University of Castilla-La Mancha. The references in brackets correspond to the designations of the strains used by Nieto-Arribas et al. [15, 16] reporting the genetic and technological characterization of these strains.

Prior to cheese-making, the freeze-dried strains were subcultured in sterilized skimmed milk and incubated for 24 h at 30 °C for *Lactococcus* strains, and for 48–72 h at 37 °C for *Lactobacillus* strain, or until they reached the stationary phase. Commercial starter Choozit™, from Danisco (Copenhagen, Denmark), containing two strains of *Lc. lactis* subsp. *lactis*, was also used.

Cheese manufacture and sampling

Cheeses were manufactured according to Manchego cheese-making technology. In all cases, 70 L of Manchega breed ewe's milk from a herd registered in the Denomination of Origin Manchego cheese was used. Ewe's milk was from the same farm over four consecutive days. Table 1 summarizes the batches manufactured and the strains used in each. Milk pasteurization was carried out by heating at 72 °C for 15 s.

Before inoculation of starter cultures, milk was tempered at 30 °C. For the CS batch, inoculation at a rate of

Table 1 Milk treatments and strains used in the manufacture of Manchego cheeses

Cheese	Milk treatment	Strains	Proportion
CS	Pasteurized	Commercial starter Choozit (two strains of *Lc. lactis* subsp. *lactis*)	Not specified
AS1	Pasteurized	*Lc. lactis* subsp. *lactis* CECT 7883 + *Lc lactis* subsp. *lactis* CECT 7884	1:1
AS2	Pasteurized	*Lc. lactis* subsp. *lactis* CECT 7883 + *Lc lactis* subsp. *lactis* CECT 7884 + *Lb. paracasei* subsp. *paracasei* CECT 7882	1:1:2
RM	Raw	No strains added	

CS commercial starter cheese, *AS1* and *AS2* autochthonous starter 1 and 2 cheeses, *RM* raw milk cheese

1.5 g/100 L was carried out following the manufacturer's instructions. Autochthonous cultures (AS1 and AS2) were added at a 1.5 % (v/v), being cell population of each strain around 10^8 cfu mL^{-1}. After 30 min, 1.6 g of bovine rennet (strength 1/150,000; 94 % chymosin and 6 % pepsin) from Rhodia (Dangé-Saint Romain, France) and $CaCl_2$ (0.02 % w/w) were added to each vat of milk and coagulation took place for 30–40 min at 30 °C. Then, the curd was cut into approximately 5 mm cubes, scalded at 38 °C for 15 min and filled into cheese moulds. Cheeses were pressed for 4 h at 15 °C, salted for 18 h in 18 % (v/v) NaCl brine at 10 °C, and ripened for 240 days at 12 °C and a relative humidity of 85 %. RM cheeses were made without addition of any starter culture and were manufactured as described above except for prior incubation of milk for 30 min.

For each batch, six cheeses of between 3 and 3.5 kg of weight were manufactured. Cheeses were ripened for 240 days. Cheese samples at 60, 150, and 240 days of ripening were taken for the different analysis. Two independent replicates of the four batches were made in consecutive weeks. The results shown constitute the mean of the two replicate trials.

Physicochemical analysis of cheeses

Dry matter (DM), fat content, and total nitrogen (TN) were analysed by the AOAC methods [18]. pH was determined by direct reading using an Ingold insertion electrode, and the salt content was analysed using a Chloride Analyser (Sherwood, model 926). All analyses were performed in duplicate.

Analysis of volatile compounds

The method used for the simultaneous distillation extraction (SDE) and the chromatographic instruments were identical to those reported by Poveda et al. [19]. SDE was carried out using 10 g of cheese, 2 mL dichloromethane as extracting solvent, and 90 μL of a camphor solution (0.5 mg mL^{-1} in absolute ethanol) as internal standard, and it was performed in duplicate with two cheese samples of each batch. Extracts were concentrated under nitrogenous stream up to a volume of 100 μL.

Peak identifications were based on comparison of their gas chromatographic retention index and their mass spectra with those of commercial pure standards from Sigma-Aldrich and with those reported by the NBS75 K and Wiley A commercial libraries. The tentative identification of compounds for which it was not possible to find reference volatiles was carried out by the comparison of their mass spectra with spectral data from the mentioned libraries, and on the basis of retention index data published in the literature [20].

The quantitative analysis was performed by total ion mode using response factors. For compounds for which commercial standards were not available, the response factors of other compounds with similar chemical structures were used. Thus, γ-caprolactone response factor was used for γ-valerolactone and γ-nonalactone quantification, benzylalcohol response factor for 3-oxo-α-ionol, and linalool response factor for fenchone and endoborneol.

Sensory analysis

Sensory analysis was carried out according to ISO 6564 [21]. The cheese samples were graded by a trained panel of 10 panellists, who had previous experience in sensory analysis of different varieties of cheeses including Manchego cheese and all were staff members of the Food Science and Technology Department in University of Castilla-La Mancha.

Olfactory sensory analysis was carried out in cheeses at 150 and 240 days of ripening. Six odour attributes were evaluated: odour intensity, odour quality, lactic acid/yoghourt odour, butter odour, ewe's milk odour, and fruity odour. These attributes were chosen by consensus from a list of terms freely generated by the panellists.

Samples were allowed to reach room temperature (20 °C) for 2 h before serving. Samples identified by 3-character codes were presented to the panellists in the form of cubes measuring 1.5 cm per side. Each panellist evaluated four samples per session. All the samples were tested twice over two different sessions.

Cheeses were evaluated using a 10-cm unstructured scale. The bottom end of the scale indicated no perceptible sensation or minimum perceptible sensation, and the top end indicated a clearly perceptible sensation. The results were then converted to numerical values (10 units).

Statistical analysis

One-way analysis of variance (ANOVA) was applied to the results of the chemical and sensory analysis using the Student–Newman–Keuls (S–N–K) test for the comparison of the means ($P < 0.05$). The statistical analysis was performed using the SPSS software package version 19.0 (SPSS Inc., Chicago, IL., USA).

Results and discussion

Physicochemical analysis of cheeses

Chemical composition of the cheeses was analysed throughout the ripening period. Table 2 shows the results for samples at 60 and 240 days. All the compositional

Table 2 Mean values ± standard deviation of chemical composition of Manchego cheeses from the different batches after 60 and 240 days of ripening

Ripening time (days)	Cheese			
	CS	AS1	AS2	RM
DM (%)				
60	59.35[d] ± 0.08	58.39[b] ± 0.03	57.71[a] ± 0.04	58.71[c] ± 0.04
240	68.37[b] ± 0.09	70.30[d] ± 0.44	69.38[c] ± 0.07	65.68[a] ± 0.04
pH				
60	4.94[c] ± 0.01	4.89[b] ± 0.01	4.84[a] ± 0.01	4.94[c] ± 0.00
240	5.00[a] ± 0.00	5.01[a] ± 0.00	5.03[b] ± 0.00	5.27[c] ± 0.00
TN (% DM)				
60	5.80[a] ± 0.02	5.83[a] ± 0.05	6.01[a] ± 0.15	5.92[a] ± 0.04
240	5.58[a] ± 0.20	5.63[a] ± 0.02	5.94[a] ± 0.30	5.82[a] ± 0.06
Salt (% moisture)				
60	3.62[a] ± 0.09	3.53[a] ± 0.07	3.49[a] ± 0.05	3.95[b] ± 0.08
240	5.93[a] ± 0.17	6.32[b] ± 0.09	5.88[a] ± 0.10	5.67[a] ± 0.19
Fat (% DM)				
60	53.91[b] ± 0.07	52.66[a] ± 0.63	53.71[b] ± 0.04	57.06[c] ± 0.03
240	55.58[c] ± 0.08	54.41[a] ± 0.16	54.77[b] ± 0.05	60.90[d] ± 0.03

DM dry matter, *TN* total nitrogen

[a–d] Means within rows without a common superscript are significantly different ($P < 0.05$) according to the Student–Newman–Keuls test

values were within the normal ranges for Manchego cheese [12] and within the limits established by the Manchego PDO Council [22].

Volatile compounds

The analysis of volatile compounds was carried out in cheeses of 240 days. A total of 58 compounds were identified (Table 3).

Free fatty acids

Free fatty acids (FFAs) were, by far, the most abundant volatile compounds in all cheeses, followed by ketones, esters, aldehydes, lactones, and alcohols (Table 3). These results agree with Gómez-Ruiz et al. [23] and with our previous findings for Manchego cheeses made with different starter strains [14].

Nine FFAs were detected in the cheeses, decanoic being the most abundant, followed by octanoic and dodecanoic acids. FFA concentration in cheese is a function of the lipolysis degree and contributes considerably to flavour formation.

Regarding pasteurized milk cheeses, CS and AS1 cheeses presented the lowest values for the total FFAs, showing AS2 cheese higher concentrations than AS1 cheese. These data are in accordance with Menéndez et al. [10] who found higher values for FFAs in Tetilla cheeses made with autochthonous lactococci and lactobacilli strains than in the cheeses made exclusively with lactococci strains. Besides, other authors found higher concentrations of FFAs in Roncal cheese made with adjunct lactobacilli and CS than in the cheese made only with the CS [24].

As expected, RM cheeses presented, except for butanoic acid, the highest ($P < 0.05$) values for FFAs. This fact may be due to a higher lipolytic activity in these cheeses, either from the native lipase activity of RM or by the more complex microbiota present. These results are in agreement with our previous findings in a study from RM and pasteurized milk Manchego cheeses [23].

The highest ($P < 0.05$) levels of 3-methylbutanoic acid, a compound perceived with high intensity in cheese by olfactometry analysis [19] and butanoic acid, a cheese-like odour compound [25], were observed in AS2 cheese. 3-Methylbutanoic acid is produced as the result of metabolism of the amino acid leucine, and butanoic acid may also be a fermentation product of lactose and lactate [26], which could be related to the presence of *Lb. paracasei* as adjunct in this cheese. These compounds may have an important influence in the sensory characteristics of this cheese, since both contribute with sweaty and strong notes. Peralta et al. [27] also found higher levels of these two compounds in hard-cooked cheese extracts inoculated with *Lb. paracasei* as compared to the control.

Ketones

Ketones were the second largest chemical family of volatile compounds in the Manchego cheeses analysed. 2-Heptanone was the main ketone found in all samples, followed by 2-nonanone and acetoin (3-hidroxy-2-butanone). CS and AS2 cheeses showed the highest concentrations of

Table 3 Volatile compounds found by SDE in Manchego cheeses from the different batches at 240 days of ripening

Volatile compound (μg g^{-1} cheese)	Cheese							
	CS		AS1		AS2		RM	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Free fatty acids								
Butanoic acid	1.467[b]	0.080	1.338[b]	0.074	2.706[c]	0.055	1.103[a]	0.100
3-Methylbutanoic acid	0.232[a]	0.044	0.488[b]	0.111	0.775[c]	0.045	0.452[b]	0.053
Hexanoic acid	4.647[b]	0.134	3.201[a]	0.509	5.408[b]	0.681	9.335[c]	0.109
Octanoic acid	12.445[b]	1.630	6.649[a]	0.888	11.356[b]	0.006	25.859[c]	1.174
Nonanoic acid	0.091[a]	0.001	0.068[a]	0.012	0.077[a]	0.013	0.134[b]	0.005
Decanoic acid	25.045[b]	1.798	15.150[a]	2.475	25.562[b]	0.187	59.230[c]	1.166
Undecanoic acid	0.178[a]	0.018	0.196[a]	0.018	0.295[a]	0.023	0.672[b]	0.018
Dodecanoic acid	5.836[a]	0.969	4.395[a]	0.182	6.769[a]	0.602	15.916[b]	0.516
Tetradecanoic acid	1.303[a]	0.251	2.174[a]	0.126	3.803[b]	0.395	7.573[c]	0.775
Total free fatty acids	51.318		33.658		56.752		120.273	
Ketones								
2-Heptanone	1.633[b]	0.180	1.346[a]	0.003	1.740[b]	0.031	1.233[a]	0.001
Acetoin (3-hydroxy-2-butanone)	0.190[a]	0.022	0.708[b]	0.061	1.265[c]	0.198	0.093[a]	0.007
2-Nonanone	0.869	0.079	0.775	0.013	0.876	0.043	0.826	0.026
2-Decanone	0.027	0.032	0.005	0.001	0.004	0.000	0.004	0.000
Total ketones	2.719		2.834		3.884		2.157	
Esters								
Ethyl lactate	0.007[b]	0.002	0.013[a]	0.003	0.030[b]	0.002	0.050[c]	0.003
Ethyl octanoate	0.071[a]	0.002	0.047[a]	0.001	0.099[a]	0.003	1.478[b]	0.062
Methyl decanoate	0.565[c]	0.052	0.358[b]	0.058	0.175[a]	0.034	0.500[c]	0.039
Methyl benzoate	0.027[b]	0.004	0.023[b]	0.006	0.012[a]	0.001	0.014[a]	0.002
Ethyl decanoate	0.333[a]	0.046	0.320[a]	0.010	0.383[a]	0.001	4.646[b]	0.389
Ethyl dodecanoate	0.133	0.036	0.091	0.017	0.084	0.018	0.142	0.008
Methyl tetradecanoate	0.006	0.001	0.010	0.000	0.020	0.014	0.021	0.004
Total esters	1.142		0.862		0.803		6.851	
Aldehydes								
Hexanal	0.066[b]	0.005	0.143[c]	0.028	0.075[b]	0.005	0.039[a]	0.004
Octanal	0.019[a]	0.003	0.043[a]	0.006	n.d.		n.d	
Nonanal	0.084	0.020	0.114	0.010	0.129	0.011	0.090	0.014
2-Octenal	0.024[a]	0.006	0.026[a]	0.001	n.d		n.d	
3-Methylthiopropanal	0.026[a]	0.004	0.040[b]	0.002	0.028[a]	0.001	0.046[b]	0.004
2-Nonenal	0.074[a]	0.019	0.139[b]	0.037	0.041[a]	0.003	0.026[a]	0.000
Decanal	0.072[a]	0.007	0.115[b]	0.003	0.105[b]	0.013	0.090[a, b]	0.007
2, 4-Nonadienal	0.019[b]	0.004	0.063[b]	0.010	0.060[b]	0.009	0.028[a]	0.003
2, 4-Decadienal	0.160[b]	0.034	0.173[b]	0.025	0.169[b]	0.017	0.010[a]	0.000
Hexadecanal	0.727[c]	0.032	0.220[a]	0.025	0.450[b]	0.013	1.127[d]	0.131
Total aldehydes	1.272		1.075		1.057		1.455	
Alcohols								
3-Methyl-1-butanol	0.073[a]	0.000	0.149[b]	0.027	0.129[b]	0.008	1.781[c]	0.031
2-Heptanol	0.068[a, b]	0.005	0.056[a]	0.005	0.093[b]	0.006	0.166[c]	0.021
3-Methyl-2-buten-1-ol	0.032[a, b]	0.007	0.022[a]	0.005	0.042[b]	0.004	0.029[a, b]	0.003
1-Hexanol	0.017[a]	0.005	0.018[b]	0.004	0.016[c]	0.005	0.033[a]	0.006
2-Ethyl-1-hexanol	0.017[a]	0.004	0.048[b]	0.003	0.027[c]	0.002	0.016[a]	0.001
1-Octanol	0.020	0.002	0.025	0.016	0.029	0.002	0.029	0.001
Nonanol + 1.3-dimethoxybenzene	0.071[a]	0.010	0.080[a]	0.022	0.081[a]	0.019	n.d.	
Decanol	0.108	0.005	0.079	0.003	0.073	0.005	0.072	0.004
Total alcohols	0.405		0.476		0.489		2.126	

Table 3 continued

Volatile compound (µg g^{-1} cheese)	Cheese							
	CS		AS1		AS2		RM	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lactones								
γ-Valerolactone[e]	0.043[a]	0.001	0.026[a]	0.000	0.036[a]	0.008	0.073[b]	0.008
γ-Caprolactone	0.166[b]	0.028	0.076[a]	0.017	0.092[a]	0.019	0.115[a]	0.008
γ-Octalactone	0.035[b]	0.007	0.010[a]	0.000	0.010[a]	0.000	0.010[a]	0.000
γ-Nonalactone[e]	0.024[a]	0.006	0.075[b]	0.006	0.105[c]	0.006	0.103[c]	0.016
δ-Decalactone	0.178[a]	0.018	0.189[a]	0.020	0.266[b]	0.011	0.252[b]	0.011
γ-Decalactone	0.144	0.006	0.150	0.005	0.240	0.011	0.204	0.006
δ-Dodecalactone	0.120[a]	0.027	0.228[b]	0.019	0.248[b,c]	0.007	0.283[c]	0.002
Total lactones	0.709		0.754		0.997		1.041	
Bencenic compounds								
Benzaldehyde	0.114[a]	0.012	0.122[a]	0.009	0.128[a]	0.009	0.197[b]	0.005
Phenylacetaldehyde	0.146[a]	0.029	0.385[b]	0.019	0.393[b]	0.030	0.165[a]	0.045
Benzylalcohol	0.193	0.017	0.336	0.080	0.441	0.036	0.388	0.040
2-Phenylethanol	0.048[a]	0.008	0.117[c]	0.011	0.076[a,b]	0.003	0.064[a,b]	0.002
Benzoic acid	0.066[a]	0.006	0.185[b]	0.042	0.238[b]	0.022	0.209[b]	0.033
Total bencenic compounds	0.566		1.145		1.275		1.023	
Terpene derivatives								
Fenchone[e]	0.032	0.006	0.027	0.011	0.028	0.002	0.040	0.001
Linalool oxide	n.d.		n.d.		0.007[a]	0.001	n.d.	
Linalool	0.010[a]	0.001	0.008[a]	0.002	0.023[b]	0.002	0.010[a]	0.002
Endoborneol[e]	0.012[a]	0.003	0.011[a]	0.001	0.018[a]	0.002	0.228[b]	0.024
Total terpene derivatives	0.054		0.047		0.076		0.278	
Miscellaneous								
Furfural	0.043[a]	0.004	0.057[a, b]	0.003	0.050[a]	0.001	0.067[b]	0.007
Heptadecane	0.043[a]	0.004	0.043[a]	0.001	0.042[a]	0.004	0.055[b]	0.002
2-Hexadecene	n.d.		n.d.		n.d.		0.052	0.006
3-Oxo-α-ionol[e]	0.020	0.004	0.015	0.000	0.016	0.002	0.017	0.001
Total miscellaneous	0.105		0.115		0.108		0.191	

Values are expressed as the mean and standard deviation calculated from the analysis by duplicate SDE extractions

n.d not detected

[a–d] Different letters in the same row indicate significant statistical differences ($P < 0.05$) according to the Student–Newman–Keuls test

[e] Compounds identified using Wiley and NBS75 K Libraries

2-heptanone. This compound, formed from the β-oxidation of some FFAs, has been related to the presence of fruity, floral, and musty notes in cheeses [25].

Concerning acetoin, it is important to underline that this compound can be produced by reduction of the butan-2,3-dione (diacetyl) or it may be synthesized from pyruvate, lactose, or citrate by lactic acid bacteria, especially *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* [28], *Leuconostoc spp,*. and enterococci [29]. It should be noted that cheeses from AS1 and AS2 batches had significantly higher ($P < 0.05$) levels of acetoin than CS cheeses. Acetoin is a pleasant aroma, and together diacetyl is related to butter aroma note.

Esters

Ethyl forms were the predominant esters in all cheeses analysed (Table 3), which is in agreement with the data reported for Manchego cheese [30]. The most abundant esters in the four batches of Manchego cheeses analysed were ethyldecanoate and methyldecanoate. In general, concentrations of esters in pasteurized milk cheeses were significantly lower ($P < 0.05$) than in RM cheese. This fact may be related to a higher esterase activity from the more complex microbiota in cheeses manufactured with RM [31]. These results agree with those of Ballesteros et al. [2] who found in raw milk Manchego cheeses a microbiota much more heterogeneous and a higher esterase (C4) and esterase-lipase (C8) activities than in pasteurized milk Manchego cheeses. Esters, especially those containing few carbon atoms, contribute in a synergistic way of giving a fruity aroma to the cheese since they have a low perception threshold value [32].

Aldehydes

Aldehydes are produced from amino acids either by transamination followed by decarboxylation or by Strecker degradation and they are easily reduced to alcohols. A total of ten aldehydes were found in the cheeses analysed (Table 3), hexadecanal being the most abundant in all the samples, followed by 2,4-decadienal, nonanal, and decanal. Again, RM cheeses accounted for the highest ($P < 0.05$) concentration of total aldehydes, followed by CS cheese. Since a high concentration of aldehydes may cause off-flavours [33], the lower concentrations that occur in cheeses with the autochtohonous strain cultures, compared with the rest of cheeses, may be considered a desirable feature in terms of cheese flavour quality.

Alcohols

The main alcohol present in all cheeses was 3-methyl-1-butanol that is believed to be produced from the reduction of 3-methylbutanal, resulting from the metabolism of leucine during cheese ripening [34]. This compound has been related to a pleasant aroma of fresh cheese and has been positively correlated with fruity odours [35]. It can be highlighted the lower concentration of 3-methyl-1-butanol in all the pasteurized milk cheeses as compared to RM cheeses. Other authors [9, 11] have reported lower concentrations of alcohols in pasteurized milk cheeses compared to RM cheeses. AS1 and AS2 cheeses showed significantly higher ($P < 0.05$) levels of 3-methyl-1-butanol than CS cheeses, probably influenced by the different starter culture used. This alcohol was also found in Pecorino Siciliano cheese made with different autochthonous strain starters, including several lactobacilli, enterococci, and lactococci [9].

Lactones

δ-Decalactone and δ-dodecalactone were among the lactones found in the highest concentrations in the cheeses. The presence of these compounds, associated with peach, apricot, and coconut odour qualities [25], could have a relevant sensory role in AS2 and RM cheeses, which presented the highest ($P < 0.05$) concentrations. Results showed that the total content of lactones in AS2 cheese, made with pasteurized milk and containing *Lb. paracasei* subsp. *paracasei* as adjunct, was almost alike to those of RM cheeses made from RM.

The presence of lactones in similar concentrations has been reported in Manchego cheese [14]. These compounds seem to be related to lipid degradation, being formed by the cyclization of γ- and δ-hydroxyacids and are considered to contribute to the buttery and fruity sensory character in cheese [19].

Bencenic compounds

Some bencenic compounds are derived from aromatic amino acids and in some cases have a relevant impact on cheese aroma [25]. The highest concentrations of these compounds were found in the cheeses manufactured with autochthonous strains.

Phenylacetaldehyde, formed by phenylalanine degradation, is one of the main bencenic compounds quantified in the analysed cheeses and has been related to honey-like and floral aromas [25]. The significantly higher ($P < 0.05$) phenylacetaldehyde concentrations that occurred in AS1 and AS2 cheeses in comparison with RM and CS cheeses could be associated with the use of the autochthonous strain starters in the manufacture of these cheeses.

Terpene derivatives

One of the most important chemical groups identified in this study are the terpenes, since they are the key odorant of several varieties of cheeses [25]. The origin of terpenes is the plants that constitute the forage mixture in pastures [36] from where they are transferred to the milk of the grazing animals and ultimately to the cheese.

RM cheeses had the highest concentration of total terpene derivatives and it is worthy to note the significantly higher ($P < 0.05$) concentration of endoborneol, also called isoborneol, which occurs in RM cheeses, although this compound can be correlated with off-flavours like camphor.

Linalool oxide was exclusively detected in AS2 cheese, probably from the hydrolysis of glycosylated forms, which may be related to the presence in these cheeses of the adjunct strain *Lb. paracasei* subsp. *paracasei*. The presence of linalool oxide and the higher ($P < 0.05$) concentration of linalool (both compounds conferring floral notes) found in AS2 cheese display the suitability of its starter and adjunct culture.

Sensory analysis

Table 4 shows the mean sensory scores awarded by the panellists at cheeses ripened for 240 days.

For the attributes odour intensity, butter odour, ewe's milk odour, and odour quality, a trend to increase during the ripening time was observed (results not shown), while

Table 4 Average odour sensory scores ± standard deviations awarded by the panellists to the 240-day Manchego cheese samples from the different batches

Attribute	Cheese			
	CS	AS1	AS2	RM
Odour intensity	$5.8^{a} \pm 0.3$	$6.8^{b} \pm 0.1$	$7.1^{b} \pm 0.3$	$8.9^{c} \pm 0.1$
Lactic acid/yoghourt odour	$2.0^{b} \pm 0.5$	$2.1^{b} \pm 0.4$	$1.6^{b} \pm 0.5$	$0.0^{a} \pm 0.0$
Butter odour	$3.4^{a} \pm 0.2$	$3.5^{a} \pm 0.2$	$4.6^{a,b} \pm 0.4$	$5.7^{b} \pm 0.8$
Ewe's milk odour	$1.9^{a} \pm 0.1$	$1.6^{a} \pm 0.2$	$3.5^{b} \pm 0.0$	$6.4^{c} \pm 0.3$
Fruity odour	$2.7^{b} \pm 0.6$	$3.5^{b} \pm 0.3$	$2.2^{b} \pm 0.9$	$0.0^{a} \pm 0.0$
Odour quality	$6.9^{b} \pm 0.2$	$7.1^{b,c} \pm 0.0$	$7.4^{c} \pm 0.1$	$6.25^{a} \pm 0.2$

a–c Means within rows without a common superscript are significantly different ($P < 0.05$) according to the Student–Newman–Keuls test

the attributes lactic acid/yoghourt and the fruity odour decreased as ripening progressed.

At 240 days, the samples, although differing in their sensory profiles, received high scores for the attributes odour intensity and odour quality, and presented moderate intensity for the butter aroma.

The scores for the attribute odour intensity of autochthonous starter cheeses were comprised between those of CS cheeses, which received the lowest ($P < 0.05$) punctuation, and RM cheeses, which received the highest ($P < 0.05$) score for this attribute. The use of the autochthonous strains seems to increase the odour intensity of the cheeses compared to the use of the CS. Among the cheeses manufactured with pasteurized milk, AS2 cheese had the highest ($P < 0.05$) scores for the odour intensity. This fact reveals the influence of the adjunct culture used in the manufacture of this batch of cheese. Related to the odour quality, the highest ($P < 0.05$) scores were assigned to AS2 cheese, followed by AS1 cheese.

The panellists only perceived the attribute lactic acid/yoghourt odour in the pasteurized milk cheeses. This attribute is primarily related to the acetaldehyde concentration, but also to the presence of other compounds such as diacetyl, acetone, and acetoin [37]. The autochthonous strain starter cheeses presented the highest ($P < 0.05$) concentrations of acetoin (Table 3), which could be related to the presence of this sensory note. These results are in agreement with Gómez-Ruiz et al. [23] who found higher values for the yoghourt odour in pasteurized milk Manchego cheeses than for the RM cheeses. Lactic acid/yoghourt odour might also be related with lactic acid and with the significantly lower pH ($P < 0.05$) in the pasteurized milk cheeses at 240 days of ripening in comparison with RM cheese (Table 1).

Butter odour was detected in all cheeses, especially in RM and AS2 cheeses. This sensory descriptor is mainly related to the presence of diacetyl and acetoin but also with δ-decalactone and δ-dodecalactone, both compounds associated with sweet, dairy, cheese, cream, and buttery aromas. These lactones were found in high concentrations in AS2 and RM cheeses.

The highest ($P < 0.05$) scores for the attribute ewe's milk odour were assigned to RM and AS2 cheeses, in this order, while CS and AS1 cheeses accounted for very low values. As expected for cheeses manufactured with raw milk, RM cheese showed the highest intensity in this attribute. This cheese presented considerably high concentrations of short-chain FFAs, which have been correlated to the odour descriptors cheese, fatty, sweat, and rancid [19] and could have been perceived by the panellists as a more intense ewe's milk odour. The presence of the adjunct in AS2 cheese could be responsible for a more intense lipolysis in this cheese compared to the other pasteurized milk cheeses, and therefore, for a higher score for the attribute ewe's milk.

Fruity odour was detected in all cheeses, except in RM cheese, but no significant differences were found among them. This attribute is related to the presence of ethyl esters. Despite the higher ($P < 0.05$) contents of ethyl esters in RM cheese, it did not necessarily display fruity aromas since these aromas may have been masked by the dominant notes of FFAs found in RM samples.

Nevertheless, it should be taken into account that it is difficult to associate the odour descriptor of an individual volatile compound with a sensory attribute, since this is perceived as the result of the interaction of a wide range of compounds in different concentrations in the cheese matrix, which give rise to an overall aroma sensation that cannot be easily predicted using individual volatile compound descriptors.

Conclusions

Manchego cheese manufactured with two autochthonous strains of *Lc. lactis* subsp. *lactis* showed, in general, similar volatile profile and odour sensory characteristics to the cheeses made with the CS culture.

However, the use of an autochthonous strain of *Lb. paracasei* subsp. *paracasei* as adjunct of the two lactococci allowed obtaining cheeses with higher ($P < 0.05$) amounts of some FFAs and alcohols, acetoin, lactones, phenylacetaldehyde, 2-phenylethanol, and linalool, than the CS cheese. Moreover, cheeses manufactured with the adjunct strain accounted for higher ($P < 0.05$) scores of the attributes odour intensity, odour quality, and ewe's milk odour than cheeses made with the CS.

In this context, it can be concluded that the use of the autochthonous strain *Lb. paracasei* subsp. *paracasei* (CECT 7882) used as an adjunct of the autochthonous strains of *Lc. lactis* subsp. *lactis* (CECT 7883 and 7884), for the manufacture of pasteurized milk Manchego cheese, results in an intensification and improvement of the aroma of the cheese and allows to obtain cheeses with odour attributes more similar to the RM traditional ones.

Acknowledgments This research was financed by the Consejería de Educación y Ciencia de la Junta de Comunidades de Castilla-La Mancha (Castilla-La Mancha Regional Government, Department of Education and Science, Project PBI08-0181-8719). The authors wish to dedicate this paper in memoriam of their colleague, the young researcher Dr. P.D. Nieto-Arribas. Author Pedro Nieto-Arribas was given a grant by the Consejería de Educación y Ciencia de la Junta de Comunidades de Castilla-La Mancha.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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