Effects of autochthonous *Kluyveromyces lactis* and commercial *Enterococcus faecium* adjunct cultures on the volatile profile and the sensory characteristics of short-ripened acid-curd Cebreiro cheese

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ABSTRACT

Four batches of Cebreiro-type cheese were made in duplicate from pasteurized milk. A control batch was manufactured with only a commercial O-starter. The other three batches were made with the same starter plus: (i) a commercial culture of *Enterococcus faecium*; (ii) a selected *Kluyveromyces lactis* adjunct culture used in a cheese-milk pre-ripening step; and (iii) the combination of both adjunct cultures. The cheeses made with the yeast adjunct were characterized by higher values of overall proteolysis, pH and a_w, and showed total and lactic acid bacteria (LAB) counts at least 2 log units than the batches made with only LAB. The volatile profiles of the cheeses made with added *K. lactis* were distinguished by high contents of esters, branched-chain alcohols, fatty acids, acetoin and 2-pnenylethanol. These batches had a more friable and sticky texture, and exhibited differential piquant, yeasty, alcoholic, acetic and fruity flavors. Furthermore, the addition of enterococci seemed to help achieve more desirable sensory characteristics. The batches manufactured with both adjunct cultures were awarded the highest scores for texture preference, flavor intensity, flavor preference, and overall sensory preference. The sensory profiles of the cheeses made with added yeast closely resembled those of traditional 'good quality' raw-milk Cebreiro cheese.

1. Introduction

The use of selected adjunct microbial cultures may improve the typicality and achieve greater differentiation of Protected Designation of Origin (PDO) cheeses, bringing their sensory quality closer to that of traditional products (Garabal, 2007). Classically, most of the efforts in this regard have focused on the selection of autochthonous lactic acid bacteria (particularly lactococci, lactobacilli and enterococci), but in recent years attention has begun to turn to indigenous yeasts. Due to their preferential growth under aerobic conditions, these microorganisms could play a major role in the ripening and formation of the characteristic sensory profiles of soft or semi-soft cheese varieties with washed or bloomy rinds, as well as fresh or short-ripened open-textured cheeses, or blue cheeses. The use of yeasts as adjunct cultures in the production of these PDO cheeses could help preserve their unique sensory characteristics, and add quality and consistency to these varieties of cheese by reducing variability in the final product (De Freitas et al., 2009; Price et al., 2014; Merchán et al., 2022).

Cebreiro is a traditional cheese variety made since ancient times in

Galicia, an Atlantic region located in northwestern Spain that produces 40% of the cow's milk collected in the country (approx. 2900 million liters in 2020; Anon, 2022). Industrial cheese is currently marketed under the Registry of the PDO Cebreiro (European Commission, 2008). The cheese is made with a curdling step characterized by a strong acid component. Cebreiro has a particular mushroom or chef's hat shape, and can be consumed either fresh or ripened. Centeno et al. (1996, 1999) concluded that a multiple-strain culture for the manufacture of Cebreiro cheese from pasteurized milk should contain selected *Enterococcus* strains with moderate proteolytic and lipolytic activities in order to reproduce the typical sensory characteristics of traditional raw-milk cheeses. According to these authors (Centeno et al., 1999), the use of enterococci would also contribute to maintaining regularity in the production and quality of Cebreiro cheeses. More recently, Atanassova et al. (2016) suggested the use of selected indigenous strains of the yeast *Kluyveromyces lactis* as adjunct cultures or co-starters in the production of PDO Cebreiro. This yeast could participate in acidification and coagulation, and the formation of gas from lactose would not be a problem because of the friable texture of the cheese. According to the

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aforementioned authors, the production of volatile compounds such as branched-chain aldehydes and alcohols, and acetic acid esters by this yeast would contribute to the formation of aromatic nuances typical of traditional fresh or short-ripened Cebreiro cheeses.

When designing the experimental cheese trials carried out in this study, it has been decided to use a commercial starter culture made up of acidifying lactococci, and a commercial (approved) *Enterococcus faecium* adjunct culture. This choice was based on the susceptibility of wild or indigenous lactococcal strains to bacteriophage infections, very common in dairies (Garneau and Moineau, 2011), and the absence of the "Generally Recognized As Safe" (GRAS, USA) or "Qualified Presumption of Safety" (QPS, Europe) grade of the *Enterococcus* genus due to its controversial epidemiological status (Hanchi et al., 2018; Terzić-Vidojević et al., 2021). Considering all the information previously exposed, the aims of the present study were: (i) to evaluate the effects of adjunct cultures of an autochthonous *K. lactis* selected strain and a commercial *E. faecium* strain on the physicochemical parameters, volatile profile and sensory characteristics of experimental short-ripened Cebreiro-type cheeses made with pasteurized milk; and (ii) to compare the volatile and the sensory profiles of the experimental cheeses with those previously described for the industrial PDO cheese and the traditional 'good quality' raw-milk product.

2. Materials and methods

2.1. Microbial cultures

The commercial starter used in the manufacture of the experimental Cebreiro cheeses was the freeze-dried direct vat set (FD-DVS) R-703 pHage Control™ (Chr. Hansen, Hørsholm, Denmark), a mesophilic type O starter containing *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains. The commercial *Enterococcus* adjunct was the freeze-dried concentrate *Enterococcus faecium* Ferlac (Abiasa, Pontevedra, Spain), described by the manufacturer as a low acidifying, highly proteolytic and aromatic culture. Freeze-dried commercial cultures of lactic acid bacteria (lactococci R-703 and enterococci Ferlac) were kept at −25 °C until use.

The selected yeast strain was *Kluyveromyces lactis* LV19TAO, a non-haemolytic strain isolated from a 7-day-old Cebreiro raw-milk cheese investigated in a previous study (Atanassova et al., 2016). The strain was characterized by weak acidifying activity and slight production of CO_2 in pasteurized whole milk, as well as the ability to generate aroma compounds in milk, particularly alcohols, acetic acid and esters responsible for fruity, alcoholic, acetic and wine-cellar nuances.

The yeast culture was inoculated at 2% in sterile (110 °C, 15 min) supplemented milk consisting of reconstituted (10%, w/v) skim milk, tryptone (5 g/L) and yeast extract (5 g/L) (all components from Oxoid, Basingstoke, UK), and incubated at 30 °C for 48 h. The supplemented milk culture was inoculated at 1% (v/v) in 500 mL of commercial high-pasteurized (90 °C, 30 s) whole (3.5 g/100 mL fat) cow's milk (Leyma, A Coruña, Spain), and incubated at 30 °C under aerobic conditions on a rotary shaker at 150 rpm for 36 h. The pasteurized-milk culture was used to inoculate cheese milk for a pre-ripening step. The absence of anti-microbial activity of the different microbial cultures against each other was confirmed by the agar well diffusion assay (data not shown).

2.2. Experimental cebreiro cheesemaking

A total of 50 L organic whole milk (4.5% fat) obtained in spring from a cattle farm with an extensively managed Brown Alpine herd fed higher proportions of pasture, was pasteurized (74 °C, 15 s) and cooled to 28 °C. The milk was divided into four 12 L automatic minivats (Perinox, Albacete, Spain) to make four different batches of cheese. A control batch was made with the only R-703 starter culture added to the cheese milk at a ratio of 10 direct culture units (U) per 100 L (1.2 DCU per 12 L of milk). A second batch (EF batch) was made with the addition of 6.5 U

of R-703 starter and 3.5 U of *E. faecium* Ferlac adjunct culture per 100 L of cheese milk (0,78 and 0,42 U per 12 L of milk, respectively). The other two batches (KL and EF + KL batches) were made with the same commercial freeze-dried cultures inoculated in identical proportions, i.e. KL as the control batch and EF + KL as the EF batch, after a pre-ripening of the cheese milk with the adjunct milk-culture of *K. lactis* LV19TAO. The yeast culture was added at a 2% ratio (240 mL per 12 L of cheese milk), in order to achieve an inoculation level of 5.5–6 log cfu/mL, and maintained in agitation (20 rpm blade rotation speed) at 28 ± 0.5 °C for 60 min.

The acidification-coagulation step was carried out at 28 ± 0.5 °C. Fifteen minutes after the addition of the freeze-dried cultures, 0.6 mL (0.05 mL per liter of milk) of a commercial chymosin coagulant (CHY-MAX® Plus, Chr. Hansen, 200 international milk-clotting units or IMCU per mL) were added to each of the minivats, homogenizing the contents by stirring (30 rpm, 1 min). Every 30 min, the pH of the cheese milks or curds, and the Dornic acidity of the cheese milks or wheys were determined until the acidification-coagulation step was finished (pH 4.60–4.65). The pH was measured using a portable digital pH meter equipped with a penetration electrode (Crison mod. 507, Barcelona, Spain). All batches were made as recommended by the Standard Procedures of the Cebreiro PDO (Xunta de Galicia, 2020). Once the milk had curdled (pH ~5.5), four cross cuts delimiting nine blocks were made in each of the curds with a sterilized broad-bladed knife. In order to evaluate possible differences in cheese yield, samples of about 50 mL of whey were taken from each of the vats after performing this operation, as well as at the end of the acidification-coagulation and cutting steps (cut up to portions of approximately 2 cm edge), and after the (12–14 h) draining stage in cloth bags (collection of 9.6 L of whey, i.e. 80% of the initial volume of milk). The drained curds were removed from the bags, salted by adding 1 g of fine salt per liter of cheese milk (12 g per batch), and manually kneaded for 5 min using disposable latex gloves. From each batch, three cheeses weighing between 0.5 and 0.6 kg were obtained. All cheeses were ripened under the same conditions (4 ± 1 °C; 90–95% relative humidity). Two cheesemaking trials were carried out using mixed milk (milk from two milkings maintained at 4 ± 1 °C for up to 16 h) from the same cooling tank collected on two different days.

2.3. Cheese sampling

The cheese batches were sampled on days 1 (only for microbiological analysis and determination of water activity) and 14 of ripening. Samples for compositional, overall proteolysis and water activity (a_w) analyses were taken from the cheese interior by previously separating a wedge of one cheese from each batch with a sterilized knife, discarding parts less than 2 cm from the surface. Ten g of the same cheese (including the cheese surface) were removed aseptically with a sterilized spatula and transferred to sterile blender bags for microbiological analyses. Cheese samples for compositional and overall proteolysis assays were kept at 4 °C until analysis within 24 h, and samples for microbiological and a_w analysis were processed immediately after collection.

Another cheese from each batch was cut into two halves and used to perform color parameter determinations. One of the halves was subsequently vacuum packed in a 90 μm thick polyamide (20%) and polyethylene (70%) plastic bag and frozen at −80 °C to carry out the analysis of volatile compounds over a period of three months. The remaining cheese from each batch was reserved for sensory analysis. All compositional, microbiological and volatile compound analyses, as well as determinations of a_w and overall proteolysis were performed in duplicate for each cheesemaking trial (four samples or subsamples per analysis).

2.4. Determination of proximate composition, pH, a_w, overall proteolysis, and color parameters

Cheeses were analyzed for dry matter content, fat content, protein

content and ash content as previously described (Centeno et al., 2015). The pH of the cheeses was measured directly with the portable pH meter by sticking the penetration electrode in three different points on the upper part of one cheese from each batch until reaching the central zone. The results reported for each batch and trial were the averages of the three readings. Water activity was determined on $3 \pm 0,1$ g samples with a Fast-lab dew point hygrometer (GBX, Bourg-de-Péage, France) in accordance with the supplier's instructions. Cheese overall proteolysis was estimated on duplicate samples by the o-phthaldialdehyde (OPA) test (Church et al., 1983), as described by Picon et al. (2010), and also by determining soluble nitrogen in 5% phosphotungstic acid over total nitrogen (PTA-SN/TN) according to Centeno et al. (1999). Color parameters were measured in the two halves of a cheese from each batch using a portable Chroma Meter CR-400 colorimeter (Konica Minolta, Sensing Global Network, Osaka, Japan). Once the instrument was calibrated, three readings were taken on both: (i) a relatively flat area of the lateral surface of the cheese showing a representative homogeneous color (surface color), and (ii) a representative area of the central part of the cheese (interior color). Both surface and interior colors were defined with the coordinates L* (ligthness; from 0 to 100 units), C* (saturation or *chroma*; from 0 to 100 units), and H* (hue angle; from 0 to 359°). Parameters C* and H* were calculated using the equations provided by the Commission Internationale de l'Eclairage (CIE, 1978). The average values corresponding to the three readings made on each of the cheese halves were recorded.

2.5. Microbiological analyses

Sample preparation and serial decimal dilutions for microbiological analyses were carried out in accordance with the International Dairy Federation Standard 122C (IDF, 1996), as described in a previous work (Atanassova et al., 2016). The following microbial groups were investigated by mixing 1 mL of the corresponding dilutions with the appropriate culture media, or by spreading aliquots of 100 µL on the selective media (enterococci, and yeast and mold counts): (a) total viable mesophilic microorganisms on plate count agar (PCA) (Oxoid) incubated at 30 °C for 72 h; (b) LAB on pH 5.7 MRS agar (Oxoid) overlaid and incubated at 30 °C for 5 days; (c) enterococci on Kenner fecal agar (KFA) (Oxoid) incubated at 44 °C for 48 h; and (d) yeasts and molds on oxytetracycline-glucose-yeast extract agar (OGYEA) (Oxoid), incubated at 25 °C for 5 days.

2.6. Determination of volatile compounds

Volatile compounds were analyzed using the headspace-solid phase microextraction-gas chromatography/mass spectrometry (HS-SPME-GC/MS) technique. For HS-SPME extraction, 1 ± 0.02 g of cheese was taken from the lateral surface of the thawed sample after scrapping an outer layer of depth 2–3 mm, and transferred into a 20-mL vial (Agilent Technologies, Santa Clara, CA, USA), which was immediately screwcapped with a laminated Teflon-rubber disc. A fused-silica 10 mm fibre coated with a 50/30-mm thickness of DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) (Supelco, Bellefonte, PA, USA) was exposed into the headspace at 37 °C for 30 min. The SPME adsorbed compounds were injected into the chromatograph with splitless mode injection (helium pressure 9.59 psi) at 260 °C for 8 min. The volatile compounds were separated, identified and quantified in a 7890B GC-System gas chromatograph (Agilent Technologies) equipped with a DB-624 capillary column (30 m, 250 µm i.d., 1.4 µm film thickness; J&W Scientific, Folsom, CA, USA) and a mass selective detector 5977B MSD (Agilent Technologies), following the method described by Domínguez et al. (2019). After chromatographic analysis, data were processed with the software MassHunter Quantitative Analysis B.07.01 (Agilent Technologies). Peak detection was done with deconvolution, and integration was performed with Agile2 algorithm. Identification of volatiles was carried out by mass spectra matches (library match factor >85%) with those contained in the National Institute of Standards and Technology mass spectral library (NIST14, Gaithersburg, MD, USA). The abundances of the identified compounds were expressed as peak area units $\times 10^{-4}$ values. Two independent analytical replicates were prepared for all cheese samples and the results were averaged for each of the trials.

2.7. Sensory analysis

Sensory evaluation was conducted in a tasting room by a panel of six connoisseurs of traditional raw-milk Cebreiro cheese and experienced in evaluation of pasteurized-milk PDO Galician cheeses. The panel included four men and two women aged between 35 and 60 years. Analyses were performed in two sessions (one session per trial). The cheeses were coded with numbers made up of three randomly chosen digits, equilibrated at room temperature (20 ± 2 °C) for 2 h and presented whole to the panelists. They were then cut into two halves to assess interior appearance, and later each half was cut into three wedges of similar size (75–110 g in weight). All the cheese wedges were marked with the corresponding numbers and delivered in random order to the panelists, in individual booths under white light according to the International Organization for Standardization 8589:2007 guidance (ISO, 2007). The panelists were given unsalted crackers, Granny Smith green apple segments and mineral water in order to cleanse, degrease and rinse their palates between tasting the different samples. The following categories and attributes were scored: (i) appearance: surface color intensity, interior color intensity, paste graininess, preference; (ii) texture: elasticity (tactile), deformability (tactile), firmness, friability, stickiness, pastiness, preference; and (iii) flavor: acid, yogurt, buttery, bitter, metallic, rancid, piquant, yeasty, alcoholic, acetic, fruity, (overall) intensity, persistence, preference. The texture attributes perceived in the mouth were basically evaluated following the indications of Lavanchy et al. (1993). Flavor (olfactory-gustatory sensations) was evaluated by chewing, salivating and swallowing the cheese paste in order to allow olfactory perception by retronasal smell. The flavor attributes were chosen based on Cebreiro PDO Regulations and the odors generated in whole milk by the microorganisms used. Each of the sensory attributes was scored on a scale of 1–7 (integers), where score 1 corresponded to the lowest degree of perception or non-perception, or "dislike very much" for preference, and score 7 reflected the highest degree of perception, or "like very much" for preference. The results were expressed as the mean score given by the panel for each attribute and cheese. An overall (preference) score was obtained by adding the weighted scores for appearance, texture and flavor preferences, multiplying the mean values by 1, 3 and 6, respectively.

2.8. Statistical analysis

All statistical procedures were performed with the software package SPSS version 25 (IBM SPSS Inc., Chicago, IL, USA). The data obtained for all the parameters analyzed in the cheeses were assessed by one-way analysis of variance (ANOVA) and differences were considered significant at $P < 0.05$ level using Tukey's test. All variables determined in 14-day-old cheeses were also subjected to a correlation analysis using a bivariate correlation test, and Pearson's coefficients were calculated with differences declared significant at $P < 0.05$ and $P < 0.01$ levels. Finally, the main sensory parameters showing statistical differences among the batches and the selected traits correlated with them were subjected to Principal Component Analysis (PCA; Varimax rotation, two components extracted).

3. Results and discussion

3.1. Cheesemaking parameters

Evolutions of pH (milk and curd) and Dornic acidity (milk and whey) throughout the manufacture of the experimental batches of Cebreiro-
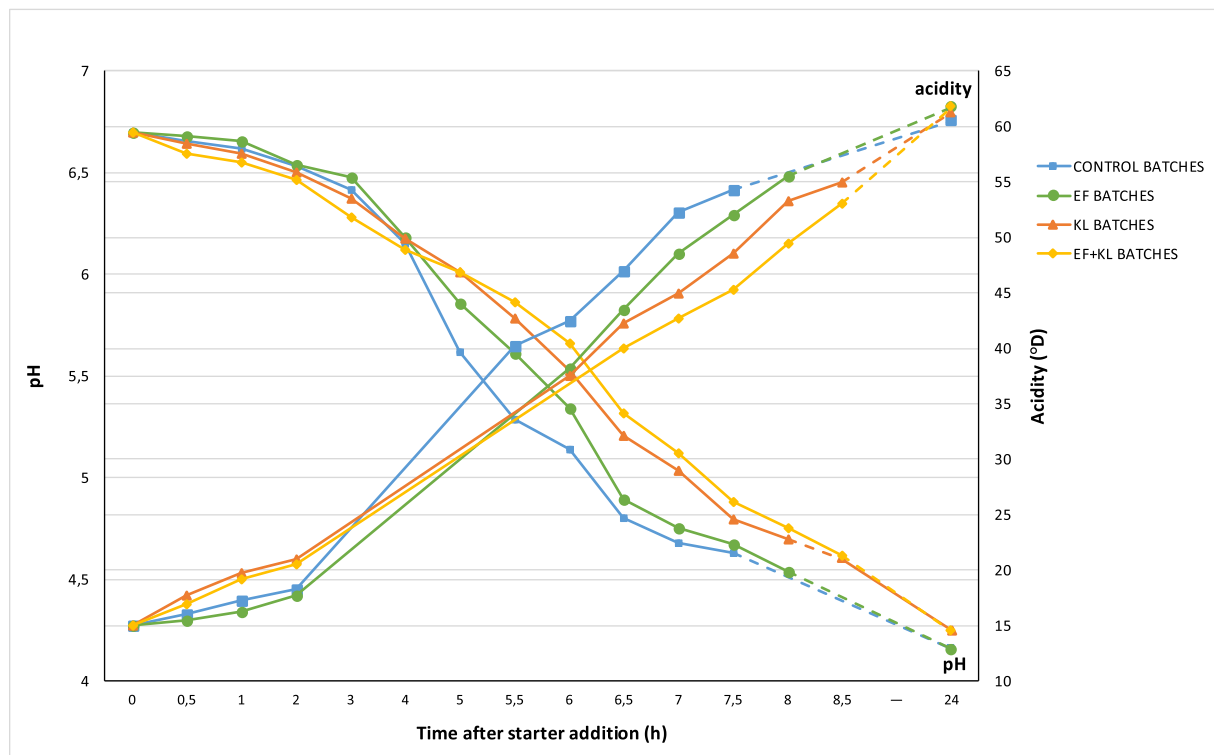
Fig. 1. Evolution of pH and total acidity (expressed as °Dornic) (mean values; n = 4) during the manufacture of Cebreiro-type cheeses made with *K. lactis* and *E. faecium* adjunct cultures.

type cheeses are shown in Fig. 1. The target final pH (4.60–4.65) was reached at 7.5 h after the addition of the starter culture in the control batches (mean value of 4.63), while the acidification rate for the EF batches was slightly slower (mean pH of 4.68 at 7.5 h). The cheeses made with the *K. lactis* adjunct achieved the final pH after 8.5 h (means of 4.60 for KL batches and 4.62 for EF + KL batches). The average acidities of the whey at the end of the acidification-coagulation step were between 53 °D for the EF + KL batches and 55.5 °D for the EF batches. Co-inoculation of *K. lactis* with starter lactococci could be expected to delay acidification as a consequence of competition for available carbohydrates in the medium. On another note, no significant differences were found at $P < 0.05$ level for the dry matters of the whey samples taken from the different batches (see Supplementary Table S1), suggesting that production yields were not affected by the inclusion of the adjunct cultures.

3.2. Proximate composition, cheese overall proteolysis and physicochemical analyses

All batches of cheese met the composition requirements specified in Cebreiro PDO Regulations (Xunta de Galicia, 2020), and no significant batch-to-batch differences were observed for any of the compositional parameters analyzed (Table 1). Other authors have reported that the addition of *K. lactis* adjunct cultures to cheese milk does not affect the proximate composition of the cheese or other chemical parameters (De Freitas et al., 2008; Kesenkaş and Akbulut, 2008). The mean OPA values in the cheeses made with the addition of *K. lactis* (0.514 for KL batches and 0.399 for EF + KL batches) were significantly ($P < 0.05$) higher than those found in the control and EF cheeses (Table 1). These values are also higher than those previously determined in 28-day-old Tetilla-type cheeses made with a proteolytic *Lactobacillus brevis* adjunct culture (mean of 0.376) (Belkheir et al., 2020). A highly positive ($P < 0.01$; r = 0.858; see Supplementary File S2 showing correlations between variables) correlation was found between OPA and PTA-SN/TN values. Strains of *Kluyveromyces lactis* have been reported to display

extracellular proteolytic activity and aminopeptidase activities stronger than those of LAB (Klein et al., 2002; Merchán et al., 2022).

The results obtained in the determination of color parameters are shown in Table 1. The mean values on the surface for the coordinates L* (80.8) and H* (99.5°) were significantly ($P < 0.05$) lower, and mean C* values (42.3) significantly higher in the KL cheeses than in the other batches. The pattern was repeated in the interior of the cheeses, although no significant differences were found between the two batch groups made with the addition of yeasts. The less light, more saturated and lower hue colors determined in the cheeses made with yeast adjuncts, particularly in KL cheeses, could perhaps be attributed to the production of brown pigments by the inoculated yeasts. Gardini et al. (2006) described a *K. lactis* strain isolated from ewe cheese able to produce pigment on cheese agar supplemented with tyrosine, a substrate that can be oxidized to melanin in reactions catalyzed by tyrosinase. For other yeast species, such as *Candida catenulata* or *Yarrowia lipolytica*, it has been reported that higher pH values and humidity intensify proteolytic activity, causing greater levels of tyrosine and strengthening the browning reaction (Nichol et al., 1996; Carreira et al., 1998).

Data recorded for pH and a_w determinations are displayed in Table 2. The pH values in the control and EF batches remained constant (means of 4.16–4.17) throughout ripening, while the pH of the cheeses made with the yeast adjuncts underwent a significant rise (from 4.25 to 5.07, for both KL and EF + KL batches). These pH increases must be related to the consumption of lactate by the yeast, in addition to the formation of alkaline products originating from proteolysis (Kesenkaş and Akbulut, 2008; Padilla et al., 2014). Water activity values decreased in all batches throughout ripening. On day 14, the values were significantly ($P < 0.05$) higher in the EF + KL batches (mean of 0.991) than in the control and EF batches (means of 0.988 and 0.987, respectively). A significant positive correlation ($P < 0.01$; r = 0.847) was found between pH and a_w values at 14 days, a fact that could be attributed to a greater whey drainage in more acidic cheeses.

Table 1

Proximate composition, overall proteolysis, and color parameters (mean ± standard deviation; n = 4) determined in 14-day-old Cebreiro-type cheeses made with *K. lactis* and *E. faecium* adjunct cultures.

	CONTROL	+ *E. faecium* FERLAC (EF)	+ *K. lactis* LV19TAO (KL)	+ EF + KL	*P* value
Dry matter (%, w/w)	47.0 ± 0.74	46.8 ± 0.08	46.9 ± 0.87	46.9 ± 0.30	0.986
Fat/dry matter (%, w/w)	58.5 ± 0.34	58.6 ± 0.51	58.6 ± 0.11	58.5 ± 0.97	0.998
Protein/dry matter (%, w/w)	34.4 ± 0.15	34.3 ± 0.54	34.2 ± 0.14	34.4 ± 0.70	0.961
Ash (%, w/w)	1.00 ± 0.06	0.97 ± 0.01	1.10 ± 0.16	1.22 ± 0.13	0.208
Moisture in fat-free basis (%)	73.1 ± 0.75	73.3 ± 0.18	73.2 ± 0.63	73.2 ± 0.22	0.980
OPA index[a]	0.188 ± 0.029[C]	0.225 ± 0.026[C]	0.514 ± 0.040[A]	0.399 ± 0.034[B]	0.000
PTA-SN/TN (%)[b]	0.35 ± 0.01[C]	0.52 ± 0.04[B]	0.80 ± 0.09[A]	0.73 ± 0.08[A]	0.000
L*_surface	92.0 ± 0.37[A]	90.5 ± 1.89[A]	80.8 ± 2.18[B]	88.8 ± 0.07[A]	0.005
C*_surface	28.6 ± 1.36[C]	30.6 ± 1.07[BC]	42.3 ± 0.71[A]	32.9 ± 0.85[B]	0.001
H*_surface (°)	105.8 ± 0.58[A]	104.5 ± 0.64[AB]	99.5 ± 0.77[C]	102.5 ± 0.30[AB]	0.002
L*_interior	92.5 ± 0.43[A]	92.9 ± 0.42[A]	87.6 ± 1.83[B]	91.2 ± 0.04[AB]	0.016
C*_interior	28.7 ± 0.22[B]	27.4 ± 0.27[B]	30.1 ± 0.59[A]	28.7 ± 0.10[AB]	0.007
H*_interior (°)	105.4 ± 0.37[AB]	106.0 ± 0.07[A]	104.2 ± 0.22[C]	105.0 ± 0.04[BC]	0.005

[A–C]Mean values within a row with different superscripts are significantly different (*P* < 0.05; Tukey's test).

[a] Expressed as absorbance units at 340 nm.

[b] Soluble nitrogen in 5% phosphotungstic acid expressed as % over total nitrogen.

3.3. Microbiological analyses

Microbial counts determined in the experimental cheeses are summarised in Table 2. On day 1, mean numbers of total mesophilic microorganisms and LAB were around 10 log cfu/g, with LAB counts being significantly (*P* < 0.05) higher in the EF batches than in the EF + KL batches. By contrast, both total and LAB counts at 14 days were significantly higher in the cheeses made with the addition of *K. lactis* (means of 9.2–9.4 log cfu/g) than in the control and EF batches (6.5–7.3 log cfu/g). These differences, of at least 2 log units, must be attributed to a stimulating effect of the yeasts on the growth of LAB. Regarding the cheeses made with added *E. faecium*, enterococci numbers were significantly higher in the EF + KL batches than in the EF batches, both on days 1 (8.7 log cfu/g vs. 7.0 log cfu/g) and 14 (9.0 log cfu/g vs. 5.0 log cfu/g). The results suggest a noticeable stimulation of the growth of the *E. faecium* Ferlac strain by the *K. lactis* LV19TAO strain. Finally, yeast and mold counts were significantly higher in the cheeses made with added *K. lactis* than in the control and EF batches, in both 1-day (means of 8.2–8.3 log cfu/g vs. 2.9–3.0 log cfu/g) and 14-day-old cheeses (9.0–9.1 log cfu/g vs. 3.8–4.3 log cfu/g). In agreement with the results obtained in the present study, De Freitas et al. (2008, 2009) associated the presence of viable *K. lactis* cells in cow's milk cheeses with prolonged survival and higher counts of starter lactococci. This same synergistic effect between *K. lactis* and LAB species has also been verified in cheese slurries (Arfi et al., 2004). The phenomenon could be attributed to the release of growth factors, such as amino acids and vitamins, by viable

Table 2

Microbial counts (log cfu/g), pH and a$_w$ (mean ± standard deviation; n = 4) determined in Cebreiro-type cheeses made with *K. lactis* and *E. faecium* adjunct cultures (samples for microbiological analyses included the cheese surface).

	CONTROL	+ *E. faecium* FERLAC (EF)	+ *K. lactis* LV19TAO (KL)	+ EF + KL	*P* value
1-day-old					
Total viable counts	10.2 ± 0.07	10.2 ± 0.04	10.1 ± 0.03	10.1 ± 0.02	0.206
Lactic acid bacteria	10.0 ± 0.01[AB]	10.1 ± 0.02[A]	9.9 ± 0.07[AB]	9.8 ± 0.06[B]	0.017
Enterococci	2.4 ± 0.17[C]	7.0 ± 0.18[B]	2.5 ± 0.08[C]	8.7 ± 0.33[A]	0.000
Yeasts and molds	3.0 ± 0.03[B]	2.9 ± 0.06[B]	8.3 ± 0.03[A]	8.2 ± 0.08[A]	0.000
pH	4.16 ± 0.03	4.16 ± 0.05	4.25 ± 0.06	4.25 ± 0.01	0.138
a$_w$	0.993 ± 0.0002	0.994 ± 0.0006	0.992 ± 0.0007	0.993 ± 0.0004	0.168
14-day-old					
Total viable counts	6.9 ± 0.18[B]	7.3 ± 0.08[B]	9.4 ± 0.08[A]	9.3 ± 0.17[A]	0.000
Lactic acid bacteria	6.5 ± 0.28[B]	7.0 ± 0.47[B]	9.4 ± 0.08[A]	9.2 ± 0.24[A]	0.001
Enterococci	3.2 ± 0.47[BC]	5.0 ± 0.96[B]	2.1 ± 0.55[C]	9.0 ± 0.10[A]	0.001
Yeasts and molds	3.8 ± 0.79[B]	4.3 ± 0.26[B]	9.1 ± 0.07[A]	9.0 ± 0.05[A]	0.000
pH	4.16 ± 0.01[B]	4.17 ± 0.07[B]	5.07 ± 0.08[A]	5.07 ± 0.01[A]	0.000
a$_w$	0.988 ± 0.0008[B]	0.987 ± 0.0004[B]	0.989 ± 0.0006[AB]	0.991 ± 0.0004[A]	0.018

[A–D]Mean values within a row with different superscripts are significantly different (*P* < 0.05; Tukey's test).

yeasts or in the process of autolysis (Addis et al., 2001; Viljoen, 2001). In addition, the increase in pH in the cheeses made with added *K. lactis* would favor the growth of LAB, including enterococci, and other mesophilic microbiota.

3.4. Volatile compounds in the experimental cheeses

A total of 53 volatile compounds were positively identified from the chromatograms corresponding to the cheese samples analyzed by the HS-SPME-GC/MS methodology used in this study. The 34 compounds showing significant (*P* < 0.05) batch-to-batch differences are classified by chemical families in Table 3. Information on the other volatiles can be found in Supplementary File S3. The abundance of the alcohol 1-propanol was significantly higher in the KL batches than in the control cheeses, and the amounts of 2-methylpropanol and 3-methylbutanol (the most abundant compound in control and EF batches) were significantly higher in the cheeses made with added *K. lactis* than in the control batches. As in the present study, Li et al. (2022) found higher contents of 1-propanol, 3-methylbutanol and 2-phenylethanol in experimental cheeses made with added *K. lactis* than in control cheeses. The ability of *K. lactis* to generate branched-chain aldehydes and alcohols in cheese preparations and experimental cheeses has been extensively reported (Martin et al., 2002; Arfi et al., 2004; Leclercq-Perlat et al., 2004; De Freitas et al., 2008). Regarding the aromatic alcohol 2-phenylethanol, its contents were significantly higher in the KL cheeses than in the cheeses made with only LAB. 2-Phenylethanol can be produced by yeasts from phenylalanine and may impart a floral, fruity and/or fermented (wine) aroma (Molimard and Spinnler, 1996; Leclercq-Perlat et al., 2004). The abundance of the ketone 3-hydroxy-2-butanone (acetoin) was significantly higher in the cheeses made with the only *K. lactis* adjunct than in the control cheeses. The same as 2-heptanol, its likely precursor 2-heptanone was only detected in the cheeses made with added *K. lactis*. Productions of acetoin and methyl ketones in cheese curds and experimental cheeses by *K. lactis* strains have been previously reported (Martin

Table 3

Abundances (mean ± standard deviation; n = 4) expressed as peak area units × 10^{-4} of the volatile compounds showing significant differences between the Cebreiro-type cheese batches at 14 days of ripening.

	CONTROL	+ E. faecium FERLAC (EF)	+ K. lactis LV19TAO (KL)	+ EF + KL	P value
ALCOHOLS					
1-Propanol	0.9 ± 0.2[B]	1.4 ± 0.4[AB]	2.6 ± 0.5[A]	2.3 ± 0.5[AB]	0.042
2-Methylpropanol	12±3[B]	35±7[AB]	60 ± 14[A]	59 ± 14[A]	0.019
3-Methylbutanol	236 ± 19[B]	407 ± 40[AB]	620 ± 119[A]	530 ± 49[A]	0.019
2-Heptanol	nd[aB]	nd[B]	2.5 ± 0.9[AB]	5.4 ± 2.0[A]	0.022
2-Phenylethanol	0.9 ± 0.3[B]	1.4 ± 0.3[B]	31 ± 10[A]	21±7[AB]	0.016
Total branched and aromatic alcohols	249 ± 16[B]	443 ± 34[AB]	711 ± 143[A]	610 ± 28[A]	0.013
KETONES					
2-Butanone	5.7 ± 2.1[A]	2.9 ± 0.8[AB]	0.9 ± 0.2[B]	0.6 ± 0.1[B]	0.034
3-Hydroxy-2-butanone	19±3[B]	27±5[AB]	37±6[AB]	46±6[A]	0.019
2-Heptanone	nd[B]	nd[B]	10±3[A]	12±3[A]	0.008
FATTY ACIDS					
Acetic acid	0.2 ± 0.1[B]	0.7 ± 0.4[AB]	2.7 ± 0.7[A]	2.3 ± 0.8[AB]	0.036
Propanoic acid	1.8 ± 0.3[B]	3.0 ± 0.6[B]	31±8[B]	69 ± 16[A]	0.005
2-Methylpropanoic acid	nd[B]	nd[B]	14±5[A]	8.8 ± 2.7[AB]	0.018
Butanoic acid	10±2[B]	18±4[AB]	31 ± 10[AB]	37±5[A]	0.040
2-Methylbutanoic acid	0.5 ± 0.1[B]	1.9 ± 0.8[B]	33±9[AB]	85 ± 35[A]	0.026
3-Methylbutanoic acid	nd[B]	0.7 ± 0.3[B]	106 ± 36[AB]	199 ± 62[A]	0.013
Hexanoic acid	1.5 ± 0.5[B]	2.3 ± 0.4[B]	18±4[A]	29±5[A]	0.003
Octanoic Acid	nd[B]	1.4 ± 0.5[AB]	2.4 ± 0.5[AB]	4.7 ± 1.7[A]	0.031
Total fatty acids	14±3[B]	28±4[B]	238 ± 10[A]	435 ± 99[A]	0.003
ESTERS					
Methyl acetate	nd[B]	nd[B]	2.1 ± 0.6[A]	1.8 ± 0.3[A]	0.004
Ethyl acetate	12±2[B]	24±5[B]	666 ± 181[A]	451 ± 69[A]	0.006
Propyl acetate	nd[B]	nd[B]	2.2 ± 0.4[A]	2.6 ± 0.4[A]	0.001
Ethyl isobutyrate	nd[B]	nd[B]	1.3 ± 0.4[A]	1.7 ± 0.4[A]	0.007
Isobutyl acetate	1.0 ± 0.3[B]	1.3 ± 0.7[B]	113 ± 23[A]	105 ± 17[A]	0.002
Ethyl butyrate	0.4 ± 0.1[B]	0.5 ± 0.1[B]	15±3[A]	22±3[A]	0.002
Isobutyl propionate	nd[B]	nd[B]	26±7[AB]	41 ± 12[A]	0.008
2-Methylbutyl acetate	nd[B]	0.4 ± 0.1[B]	164 ± 43[A]	94 ± 39[AB]	0.012
Isoamyl acetate	6.7 ± 1.8[C]	20±4[BC]	795 ± 242[A]	549 ± 101[AB]	0.009
3-Methyl-3-butenyl acetate	nd[B]	nd[B]	2.7 ± 1.0[A]	2.2 ± 0.3[A]	0.012
Isobutyl butyrate	nd[B]	nd[B]	2.6 ± 1.2[AB]	10±4[A]	0.021
Ethyl hexanoate	nd[B]	1.2 ± 0.4[B]	22±6[A]	27±6[A]	0.006
Isoamyl isobutyrate	nd[B]	nd[B]	33±8[AB]	47 ± 17[A]	0.016
Isoamyl butyrate	nd[C]	nd[C]	17±4[B]	41±6[A]	0.001
Ethyl octanoate	nd[B]	nd[B]	7.1 ± 2.3[AB]	13±3[A]	0.006
Phenethyl acetate	3.5 ± 1.1[B]	5.2 ± 2.2[B]	237 ± 51[A]	361 ± 82[A]	0.004
Ethyl decanoate	nd[B]	nd[B]	8.0 ± 1.5[A]	7.6 ± 2.8[A]	0.010
Phenethyl butyrate	nd[B]	nd[B]	16±2[B]	97 ± 16[A]	0.001
Total esters	24±3[B]	53±3[B]	2131 ± 313[A]	1875 ± 250[A]	0.001

[A–C] Mean values within a row with different superscripts are significantly different (P < 0.05; Tukey's test).

[a] nd: Compounds not detected (considered as 0.0 values for statistical analysis).

et al., 2002; Li et al., 2022).

Eight fatty acids were identified in the cheeses analyzed in the present study, all of them showing significant (P < 0.05) batch-to-batch differences and having their highest contents in the cheeses made with added K. lactis (Table 3). The branched 2-methylpropanoic (isobutyric) acid was only detected in the cheeses made with the yeast adjunct, while propanoic, 2-methylbutanoic, 3-methylbutanoic (isovaleric), and hexanoic acids were detected in significantly higher amounts in the cheeses made with the two adjunct cultures than in the control and EF batches. Branched-chain fatty acids are characteristic compounds of goat and ewe cheeses, and have been described in cheeses and cheese-like medium inoculated with K. lactis (De Freitas et al., 2008; Padilla et al., 2014; Li et al., 2022). Octanoic acid was not detected in the control cheeses but it was in the EF batches. Enterococci seem to have a higher esterolytic activity than the remaining LAB genera (González et al., 2010; Graham et al., 2020), which appears to be mostly restricted to short-chain fatty acids. The results of the present study suggest that this action has complemented the lipolytic activity of yeasts in the release of volatile fatty acids.

A total of 18 esters were detected in the experimental cheeses, all of them present in higher amounts in the cheeses made with added yeasts and showing significant batch-to-batch differences (Table 3). Eleven ester compounds were only detected in the cheeses made with the

K. lactis adjunct: methyl acetate, propyl acetate, ethyl isobutyrate, isobutyl propionate, 3-methyl-3-butenyl (isoprenyl) acetate, isobutyl butyrate, isoamyl isobutyrate, isoamyl butyrate, ethyl octanoate, ethyl decanoate, and phenethyl butyrate. Among these, isoamyl butyrate and phenethyl butyrate were determined in significantly higher contents in the cheeses made with the two adjuncts than in the KL batches. Isoamyl acetate and ethyl acetate reached the highest abundances among all the volatiles identified in this study in KL cheeses. Ethyl acetate has been described as the main ester produced by K. lactis in cheese slurries (Arfi et al., 2004; Leclercq-Perlat et al., 2004). The branched esters isobutyl (2-methylpropyl) acetate, isoamyl (3-methylbutyl) acetate and phenetyl (2-phenylethyl) acetate, as well as propyl acetate, ethyl isobutyrate, ethyl butyrate, ethyl hexanoate and ethyl octanoate have also been identified in cheese slurries, experimental cheeses and cheese-like medium containing K. lactis (Leclercq-Perlat et al., 2004; De Freitas et al., 2008; Padilla et al., 2014; Price et al., 2014; Li et al., 2022).

The volatile profiles of the cheeses made with the K. lactis adjunct were characterized by high contents of esters (particularly acetic esters), branched-chain alcohols, fatty acids (particularly branched fatty acids), acetoin and 2-pnenylethanol. These profiles seem rather different from those of industrial Cebreiro PDO cheeses made with commercial DL-starters, with a virtual absence of fatty acids and esters and low amounts of branched aldehydes and alcohols, and with high contents of
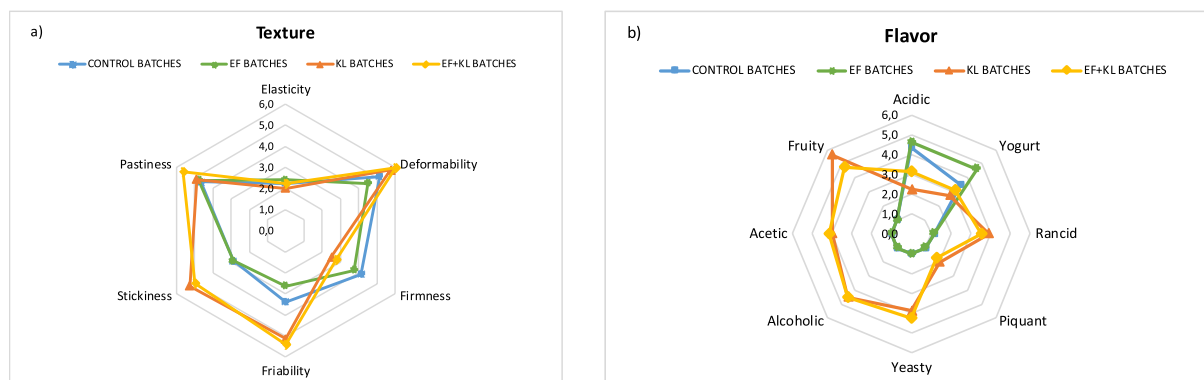
Fig. 2. Texture and flavor profiles (mean values; n = 12) of Cebreiro-type cheeses made with *K. lactis* and *E. faecium* adjunct cultures.

ethanol, isopropanol and diacetyl (Centeno et al., 2015). Regarding the main correlations between volatile abundances and microbial counts, total branched and aromatic alcohols, total fatty acids, and total esters were positively correlated at the $P < 0.01$ level with total mesophilic, LAB, and yeast and mold counts (Table S2).

3.5. Sensory analysis of the cheeses

Results obtained for the texture parameters evaluated in the cheese batches are shown in Fig. 2a. Flavor parameters for which significant ($P < 0.05$) batch-to-batch differences were detected are displayed in Fig. 2b. Other sensory data can be found in the Supplementary Table S4. No significant differences between batches were observed for elasticity and pastiness attributes. The scores obtained for firmness were significantly higher in the control batches (mean value of 4.13) than in the batches made with the *K. lactis* adjunct. On the contrary, both friability and stickiness parameters were scored with significantly mean higher values in the batches made with added yeasts (maximum of 5.38 for friability in EF + KL batches) than in the cheeses made with only LAB. The lower firmness and higher friability of the batches made with the *K. lactis* culture may be related to the production of CO_2 and the consequent formation of cavities in the curd by this fermentative yeast during cheesemaking, in addition to higher levels of casein hydrolysis in the protein network. Significant differences between batches were found for the following flavor parameters: acidic, yogurt, rancid, piquant, yeasty, alcoholic, acetic, and fruity. The last five attributes mentioned were not perceived in the cheeses made with only LAB by any of the panelists. No significant batch-to-batch differences were found for bitter flavor, despite the more intense proteolysis in the cheeses made with added yeasts. According to Curioni and Bosset (2002), ethyl acetate could contribute to minimizing the sensations of sharpness and bitterness of cheeses. Acidic and yogurt flavors received significantly higher scores in the control and EF batches (means of 3.50–4.63) than in KL cheeses, with the acidic variable being negatively correlated with pH ($P < 0.05$; r = −0.815). Rancid and piquant flavors may be attributed to the release of short-chain fatty acids and branched fatty acids. Yeasty or intense cheesy aromas could be related to 2-heptanone and short-chain fatty acids such as butanoic acid (Molimard and Spinnler, 1996; Frank et al., 2004), this attribute being highly positively ($P < 0.01$; r > 0.950) correlated with the methyl ketone and total fatty acids. "Alcohol" odor has also been described in experimental cheeses made with *K. lactis* (De Freitas et al., 2008). Finally, fruity aromas must be related to the production of esters and branched aldehydes and alcohols by *K. lactis* (Martin et al., 2002; Arfi et al., 2004; Leclercq-Perlat et al., 2004; De Freitas et al., 2008; Gkatzionis et al., 2009). The term most used by the panelists to describe the flavor of KL cheeses was "fermented grape pomace", while flavor of EF + KL cheeses was most often described as "fermented bakery dough". Price et al. (2014) mentioned the attribute "fermented apples" to describe the odor of *K. lactis* cultures in whole

Table 4
Preference, intensity and persistence (mean ± standard deviation; n = 12) for the different sensory categories determined in 14-day-old Cebreiro-type cheeses made with *K. lactis* and *E. faecium* adjunct cultures.

	CONTROL	+ *E. faecium* FERLAC (EF)	+ *K. lactis* LV19TAO (KL)	+ EF + KL	*P* value
Appearance preference	4.38 ± 1.19	4.50 ± 0.76	5.50 ± 1.07	5.50 ± 1.41	0.092
Texture preference	3.25 ± 0.89[B]	3.75 ± 0.71[B]	4.88 ± 0.64[A]	5.75 ± 0.46[A]	0.000
Flavor intensity	3.63 ± 0.52[B]	4.13 ± 0.64[B]	5.75 ± 0.46[A]	6.00 ± 0.76[A]	0.000
Flavor persistence	2.63 ± 0.52[B]	4.38 ± 0.92[A]	5.25 ± 0.71[A]	5.38 ± 0.92[A]	0.000
Flavor preference	3.25 ± 0.71[C]	4.13 ± 0.64[B]	5.75 ± 0.46[A]	6.50 ± 0.53[A]	0.000
Overall preference[a]	33.6 ± 6.0[D]	40.5 ± 5.8[C]	54.6 ± 2.6[B]	61.8 ± 3.9[A]	0.000

[A–C]Mean values within a row with different superscripts are significantly different ($P < 0.05$; Tukey's test).
[a]Calculated by multiplying the mean scores for appearance, texture and flavor preferences by 1, 3 and 6, respectively, and adding the results obtained.

milk.

Data obtained for preferences of the sensory categories, as well as for flavor intensity and persistence are shown in Table 4. The scores for texture preference, flavor intensity and flavor preference were significantly ($P < 0.05$) higher in the batches made with the *K. lactis* adjunct than in the cheeses made with only LAB. The significantly higher scores for flavor preference and persistence awarded to EF batches compared to control cheeses, and the higher flavor preference values for EF + KL batches in comparison with KL cheeses suggest a positive contribution of *E. faecium* on the sensory quality of Cebreiro-type cheeses made in the present study. This microorganism may have contributed to the enhancement of cheese flavor through its proteolytic and/or peptidase activities (Perin et al., 2017; Graham et al., 2020) in addition to its esterolytic ability, favoring the development of a more intense and balanced cheese flavor. Surprisingly, the calculated overall scores significantly differentiated the four cheese batches, with the highest mean value (61.8 out of a total of 70) corresponding to EF + KL cheeses. The appearance and flavor of control and EF batches match to a greater extent with the profiles outlined for industrial cheeses (Centeno et al., 2012) and those poorly described in Cebreiro PDO Regulations (white color; homogeneous paste; acidic, cream and yogurt flavors). However, the friable texture and the granular, melting and highly sticky mouthfeel referred to in the PDO Regulations seem to be more in line with the

Table 5

Main bivariate correlations between preferences for the sensory categories and other physicochemical parameters, microbial counts and volatile compounds determined in 14-day-old Cebreiro-type cheeses.

	Pearson's correlation coefficients[a]			
PARAMETERS	Appearance preference	Texture preference	Flavor preference	Overall preference
pH	0.998**	0.896**	0.946**	0.961**
a_w	0.857**	0.908**	0.836**	0.878**
H*_surface	−0.859**	NS	−0.716*	−0.733*
OPA index	NS	0.678**	0.807**	0.808**
Total viable counts	0.985**	0.852**	0.953**	0.953**
Lactic acid bacteria	0.978**	0.820*	0.947**	0.940**
Yeasts and molds	0.991**	0.917**	0.925**	0.952**
3-Methylbutanol	0.836**	NS	0.781*	0.776*
Total branched and aromatic alcohols	0.859**	NS	0.806*	0.803*
3-Hydroxy-2-butanone	0.866**	0.813*	0.936**	0.922**
3-Methylbutanoic acid	0.876**	0.905**	0.923**	0.938**
Total fatty acids	0.895**	0.926**	0.947**	0.961**
Ethyl acetate	0.935**	0.754*	0.824*	0.835**
Ioamyl acetate	0.933**	0.755*	0.825*	0.836**
Phenethyl acetate	0.935**	0.924**	0.953**	0.969**
Total esters	0.986**	0.852**	0.912**	0.926**

[a]NS: Non-significant correlation; *: Significant correlation at $P < 0.05$; **: Significant correlation at $P < 0.01$.

cheeses made with added yeasts. On the other hand, the sensory profiles of the batches made with the *K. lactis* adjunct closely resemble those described by Centeno et al. (2012) for 'good quality' traditional raw-milk Cebreiro cheeses aged 15–30 days. The texture of these cheeses was described as friable, granular, pasty and sticky, and the terms used to define their flavor were: buttery, slightly bitter, yeasty, slightly rancid, slightly piquant, alcoholic, kefir and fruity.

Main correlations between sensory preferences and other parameters are shown in Table 5. Overall score was highly ($P < 0.01$) positively correlated with pH, a_w, OPA index, total counts, LAB counts, yeast and mold counts, total fatty acids and total esters. Flavor intensity, flavor persistence and flavor preference were all highly positively correlated with rancid, piquant, yeasty, alcoholic, acetic and fruity attributes (File S3). The principal sensory parameters and the selected traits correlated with them were extracted in two components by the PCA method (Fig. 3). Component 1 explained 81.5% of the total variance and was highly positively ($r > 0.800$) correlated with the variables: a_w, acetoin, 2-heptanone, total fatty acids, texture preference, piquant flavor, yeasty flavor, acetic flavor, flavor preference and overall score, and highly negatively ($r = −0.773$) correlated with firmness (see Supplementary File S5 showing the rotated factor loadings). Finally, it must be taken into account that some of the volatile compounds detected in the present study, such as branched-chain fatty acids or esters such as ethyl iso-butyrate and ethyl hexanoate, could be responsible for off-flavours (putrid or overwhelming fruity flavor defect) if accumulate to higher levels over a longer ripening period.

4. Conclusions

The results of this study seem to confirm that the use of selected strains of *K. lactis* would undoubtedly contribute to an obvious sensory differentiation of Cebreiro PDO cheese. This yeast appears to enhance proteolysis and lipolysis and the formation of volatile compounds, particularly branched-chain alcohols, fatty acids and esters, and consequently modify the texture and flavor profile of Cebreiro. The addition of proteolytic enterococci (commercial *E. faecium*) also seems to help achieve a more intense and balanced flavor. When both adjunct cultures are used in combination, the cheeses obtained are very similar in their sensory profile to the traditional 'good quality' raw-milk product. Although further research through omics approaches would be highly advisable, particularly those issues concerning the interactions between *K. lactis* and LAB present in the cheese microbial community, the flavor generation pathways, and the enzymes involved in them, it appears that selected adjunct cultures of *K. lactis*, alone or in combination with adjunct enterococci, would add quality and consistency to fresh or short-ripened acid-curd cheeses such as PDO Cebreiro variety.
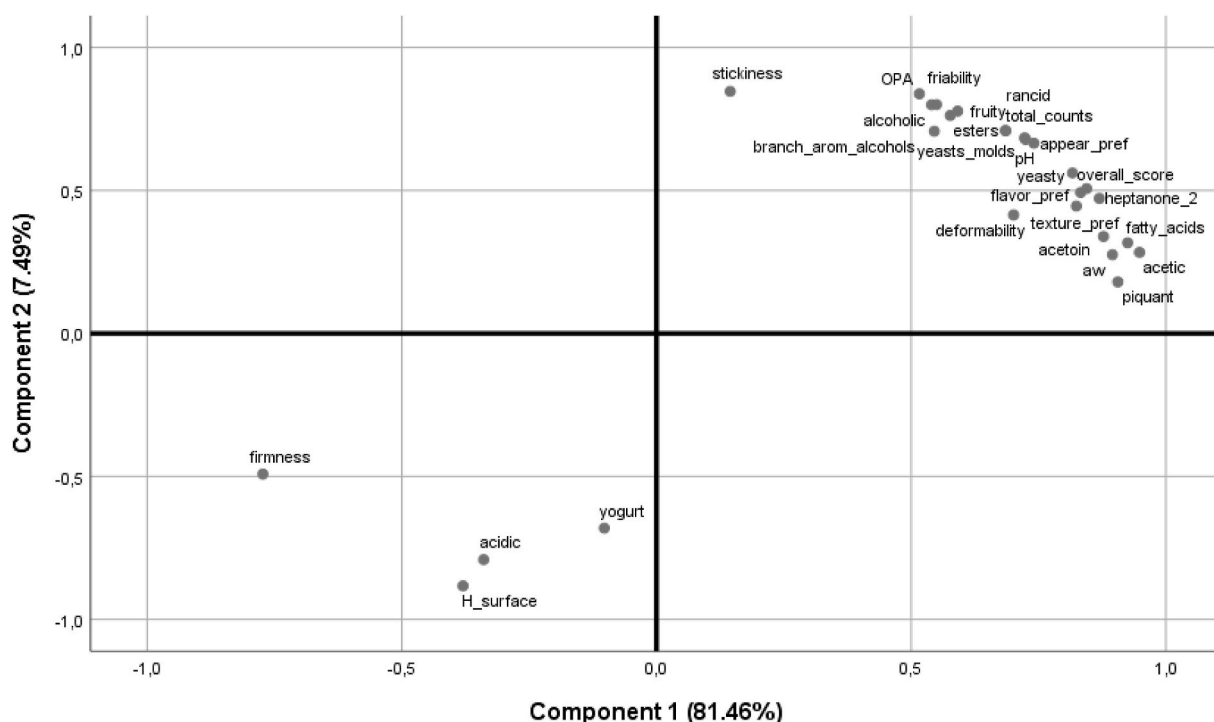

Fig. 3. Component plot in rotated space showing the variables subjected to Principal Component Analysis (the full names of some variables are shown in Tables 3–5).

Declaration of competing interest

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104101.

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