Research Article

Received: 20 June 2014 Revised: 22 December 2014 Accepted article published: 16 January 2015 Published online in Wiley Online Library: 11 February 2015

(wileyonlinelibrary.com) DOI 10.1002/jsfa.7093

Biogenic amines in Zamorano cheese: factors involved in their accumulation

Patricia Combarros-Fuertes, Domingo Fernández, Ricardo Arenas, Isabel Diezhandino, Maria Eugenia Tornadijo and José María Fresno*

Abstract

BACKGROUND: Ripened cheese is among fermented food the most often associated with food poisoning from biogenic amines. The influence of ripening time, heat treatment of milk and the effect of using milk from a different ewe breed on the biogenic amine (BA) content of Zamorano cheese was studied by high-performance liquid chromatography. Physicochemical, proteolytic and microbiological parameters were also studied.

RESULTS: BA content increased significantly during ripening and their final values were around 400 mg kg^{-1}. Cheeses elaborated with raw milk duplicated the concentration of BA relative to those elaborated with pasteurized milk (72 °C for 20 s). The average levels of putrescine, spermine and tyramine were higher in cheeses made with a greater proportion of milk from Churra breed. Significant differences in microbial counts and nitrogen soluble in 5% phosphotungstic acid were observed between the different batches.

CONCLUSION: Ripening time and heat treatment applied to milk were the factors that exercised the greatest influence upon the concentration of BA in Zamorano cheese.

Keywords: phenylethylamine; tyramine; pasteurization; ripening; microbial counts; proteolysis

INTRODUCTION

Biogenic amines (BA) are nitrogenous organic compounds of low molecular weight that originate principally from the decarboxylation of free amino acids. They are produced naturally during microbial, plant or animal metabolism.[1] BA ingested with food can be detoxified by the action of two enzymes present in gut epithelium: monoamine oxidases (MAO) and diamine oxidases (DAO). However, in some cases, they can produce adverse toxic reactions, either directly such as histamine (related to headache, nasal secretion, bronchospasm, hypotension, oedema or even anaphylactic shock)[2] and tyramine (related to hypertension, headache, perspiration, vomiting or pupil dilatation),[3] or indirectly such us putrescine and cadaverine, which can potentiate the toxic effects of other amines.[4] Furthermore, some amines can react with nitrite, encouraging the appearance of other toxic compounds such as nitrosamines.[5]

The formation of BA in foodstuffs occurs primarily through the action of bacteria during processing and storage, although there are some biogenic amines such as spermine and spermidine which are present naturally in raw matter. In addition, among fermented foods, cheese is one of the most often associated with food poisoning from biogenic amines.[3]

Zamorano cheese is a variety elaborated from an uncooked pressed curd, with a cylindrical shape and a weight of around 3 kg. Ewe's milk is used in its elaboration and its ripening time period must exceed 4 months. Its higher quality was recognized by the EU in 1996 through the concession of a Protected Designation of Origin (PDO).[6]

Currently, Zamorano PDO cheese may be produced from raw or pasteurized ewe's milk, depending on the type of cheese-making establishment (craft or industrial), using a starter culture mixture of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. At the end of ripening time, Zamorano cheese is characterized by a total solid (TS) content around 688.9 ± 15.1 g kg^{-1}, a fat content of 605.3 ± 17.8 g kg^{-1} in dry matter and a protein content around 344.2 ± 6.2 g kg^{-1} in dry matter. Salt content reaches an average final value of 32.5 ± 8.2 g kg^{-1} TS and pH values are around 5.90 ± 0.21.[7] Ripening time is also very variable, running from 4 up to 12 months. Moreover, in elaboration of Zamorano PDO cheese, the Regulatory Council allows the use of ewe's milk only from Churra and Castellana breeds. However, in recent years there has been a debate within the Regulatory Council between smaller and larger cheese-makers with regard to modifying the Regulations for this PDO so as to include Assaf breed, because of its greater productive capacity. This fact has led many producers who provide milk to the cheese industries registered with the Regulatory Council of Zamorano PDO cheese to opt for the Assaf breed, to the detriment of the Churra and Castellana breeds. These variations in the elaboration of Zamorano cheese could bring with them

* *Correspondence to: José María Fresno, Department of Food Hygiene and Technology, Faculty of Veterinary Science, University of Leon, 24071 León, Spain. E-mail: jmfreb@unileon.es*

Department of Food Hygiene and Technology, Faculty of Veterinary Science, University of Leon, 24071 León, Spain

more or less important changes in its microbiological, physical, chemical and biochemical characteristics as well as in sensory properties, with an impact on final quality.

During ripening an intense proteolysis occurs; caseins are hydrolysed to free amino acids which can be decarboxylated to BA. The formation of BA is favoured if environmental conditions during cheese ripening (temperature, pH, water activity, salt/moisture (S/M) ratio) are within the optimum range for the action of decarboxylase enzymes.[8] Decarboxylase enzymes are produced by some bacteria such as lactic acid bacteria, which are present naturally in milk, are added with the starter culture or contaminate the cheese during manufacture and/or storage. Lactobacilli usually dominate the non-starter lactic acid bacteria (NSLAB) microflora that mainly originate from the industrial environment.[9] NSLAB are involved in proteolytic and lipolytic phenomena during cheese ripening and affect the sensory characteristics of the cheese. However, it is considered that these microorganisms are important in biogenic amine formation as they have the ability to decarboxylate amino acids, as has been described by other authors.[8,10]

The European Food Security Agency (EFSA) has established a protocol for gathering data on the levels of BA present in different fermented foods and drinks in order to evaluate the risks associated with their consumption[3] and to provide a greater control of the concentration of BA in these products when they are elaborated and sold, with the aim of avoiding or minimizing risks to consumers' health. In this respect our study provides very useful information, as the aim was to evaluate the influence of ripening time, heat treatment applied to the milk (raw or pasteurized) and the type of ewe's milk used (from Churra or Assaf breed) on biogenic amine content of Zamorano PDO cheese.

MATERIAL AND METHODS

Cheese-making and sampling

The same procedure was used to elaborate all batches of cheese. Briefly, calcium chloride (0.2 g L^{-1}), potassium nitrate (0.25 g L^{-1}) and a commercial starter culture of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (0.8%, CHOOZIT™ MA 11 LYO 50 DCU; Danisco, France) was added to raw or pasteurized (72 °C for 20 s) ewe's milk at 32 °C. After half an hour, 30 mL liquid lamb rennet (80% chymosin; 20% pepsin; 75 RU; Cuajos Caporal, Valladolid, Spain) were added per 100 L of milk. Coagulation occurred in 30 min. Curd was then cut until rice grain size was achieved and stirred for a further 30 min, while slowly increasing the temperature to 36–38 °C at a rate of 1 °C every 5 min. Once the curd had acquired the optimal consistency, the mass was drained of whey and transferred to cylindrical moulds (15 cm high × 21 cm in diameter). The curd was pressed at 3.5 kg cm^{-2} for 8–10 h until a pH of 5.3 was reached and was then salted in brine (200 g NaCl kg^{-1}, temperature 8–10 °C and pH 5.3) for 24 h. Finally, cheeses were transferred to the ripening chambers of a cheese producer belonging to the Regulatory Council for Zamorano PDO cheese, where they remained for 10 months at a temperature of 8 °C and a relative humidity of 80–85%.

Two experimental procedures were used: (A) to determine the effect of heat treatment, five batches of cheese were produced with raw Churra milk and five batches with pasteurized Churra milk; (B) to evaluate the effect of milk from a different ewe breed, five batches by duplicate were produced, involving the following combinations of raw milk: 100% Churra; 75% Churra to 25% Assaf; 50% Churra to 50% Assaf; 25% Churra to 75% Assaf; and 100% Assaf. In each batch, 12 cheeses of approximately 3 kg in weight were manufactured: in total, 120 cheeses for each experimental procedure. Cheeses were sampled at 1, 60, 120, 180, 240 and 300 days of ripening. At each sampling point, two complete cheeses were taken for analysis (Fig. 1).

Physicochemical analysis

Dry matter was determined following the IDF standard 4.[11] Fat content was analysed following the IDF standard 222.[12] Protein content was determined following the macro-Kjeldahl method IDF standard 20–1,[13] using a nitrogen-to-protein conversion factor of 6.38. Water activity (a_w) was determined using an Aqua Lab CX-2 water activity system (Decagon, WA, USA). To measure the pH, 10 g of cheese were homogenized in an Omni-Mixer homogenizer (Cole-Parmer, Spain) with 100 mL distilled water at 45–50 °C (previously boiled). The mixture was filtered with a Whatman No. 40 filter (Whatman Biosystems, Maidstone, UK) and the pH was measured at 20 °C with a Micro pH 2002 pH meter (Crison, Barcelona, Spain). NaCl content was determined using the procedure reported by Volhard according to AOAC standard 935.43.[14] Lactose content was measured following the method reported by Munson-Walker according to IDF standard 43.[15]

Proteolytic analysis

Nitrogen soluble in water at pH 4.4 (pH 4.4-SN); nitrogen soluble in 12% trichloroacetic acid (TCA 12%-SN) (related to small peptides, free amino acids and ammonia, derived from rennet and microbial proteolytic enzymes action) and nitrogen soluble in 5% phosphotungstic acid (PTA 5%-SN) (related to free amino acids derived from aminopeptidase activity of microorganisms) were determined following the method described by Bütikofer *et al.*[16]

Microbiological analysis

Samples were prepared according to the IDF standard 122B.[17] Total lactic acid bacteria were counted on MRS agar after incubation at 30 °C for 72 h.[18] Enterobacteriaceae were counted on violet red bile glucose agar (VRBGA) (Oxoid) after incubation at 37 °C for 18–24 h.[19]

BA analysis

BA (tyramine, putrescine, cadaverine, spermine, tryptamine, phenylethylamine, histamine and spermidine) extraction and derivation were carried out following the method of Innocente *et al.*[20] Separation, identification and quantification of BA were carried out by reversed-phase high-performance liquid chromatography (RP-HPLC), in accordance with the methodology described by Moret *et al.*,[21] with some modifications in the elution gradient as described below.

A stock of standard solutions were prepared following the method described by Eerola *et al.*[22] by adding an accurately weighed amount of each amine (23 mg tyramine, 15.5 mg putrescine, 24 mg cadaverine, 30.9 mg spermine, 8.8 mg tryptamine, 19 mg 2-phenylethylamine, 57.5 mg histamine, 21.1 mg spermidine and 30.2 mg 1,7-diaminoheptano as internal standard) to a 25 mL volumetric flask and brought to this volume with MilliQ water. The standard solutions were stored at 4 °C. A working standard solution was prepared mixing different volumes of stock solutions in a 100 mL volumetric flask and diluted with MilliQ water to obtain a final concentration of 100 mg L^{-1} of each biogenic amine. A calibration line for each dansylated biogenic amine was obtained by analysing the working standard solution

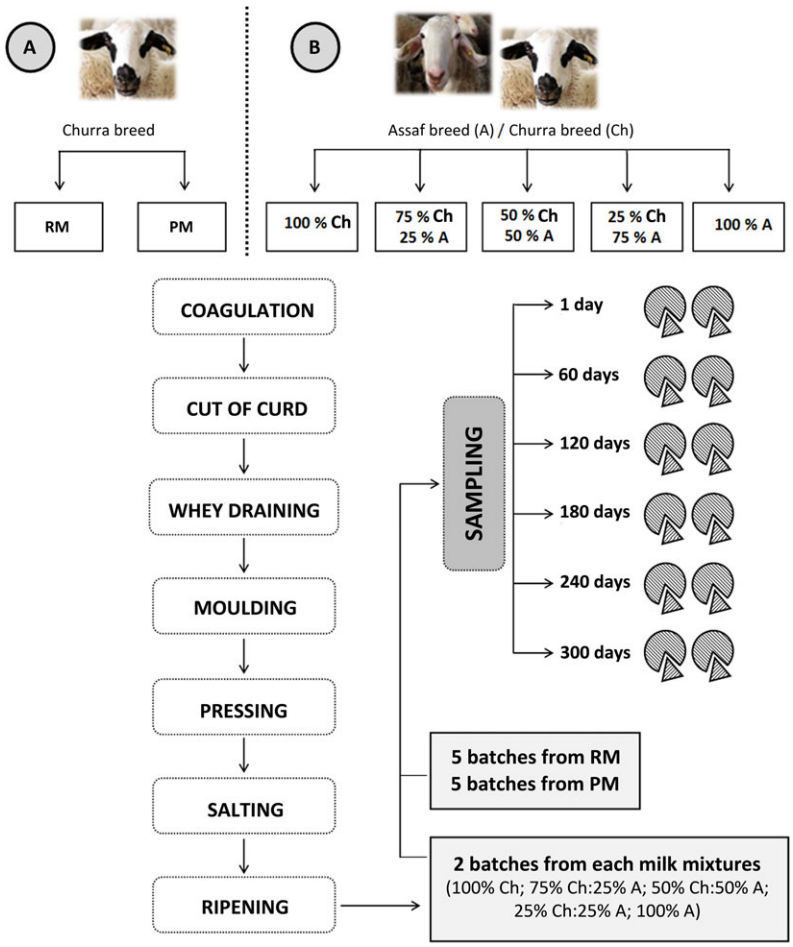

Figure 1. Cheese-making and sampling procedures for Zamorano cheese elaborated with raw milk (RM), pasteurized milk (PM) and mixtures of milk from Churra and Assaf breeds.

diluted at different concentrations.[22] Concentration of each amine used for calibration line, equation, linearity and limit of detection and quantification are shown in Table 1.

For BA extraction, 10 g cheese was homogenized with 20 mL hydrochloric acid 0.1 mol L^{-1} and 100 ~L of an internal standard solution (0.6 mg mL^{-1} 1,7-diaminoheptane) for 2 min using an Omni-Mixer (Sorwall, Newtown, CT, USA). The homogenate obtained was centrifuged at 4 °C in an Eppendorf 5804R centrifuge (Hamburg, Germany) at 15 557 × *g* for 20 min. This operation was repeated twice. The supernatant was brought to a final volume of 50 mL with 0.1 mol L^{-1} HCl.[20]

In test-tubes protected from light, 1 mL of the extract from each sample was mixed with 200 ~L of a solution of 2 mol L^{-1} sodium hydroxide, 300 ~L of a saturated solution of sodium bicarbonate and 1 mL of a solution of dansyl chloride in acetone (10 mg mL^{-1}). The tubes were tightly closed and placed in a water bath at 40 °C for 60 min. Thereafter, when they had cooled to room temperature, 100 ~L of a 25% solution of ammonium hydroxide were added and they were kept in darkness for 15 min. Next, 1 mL diethyl ether was added to each tube; they were shaken and then centrifuged at 2057 × *g* for 3 min at room temperature. The supernatant was collected in a sealed test-tube. This procedure was repeated. The

Table 1. Parameters of calibration standard solutions of biogenic amines studied: concentration range, equation of calibration curves, correlation coefficients (R^2), limit of detection (LOD) and limit of quantification (LOQ).

Biogenic amine	Concentration (mg L^{-1})	Equation	R^2	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Tryptamine	5–100	$y = 18905x + 151\,468$	0.997	2.1	4.6
Phenylethylamine	5–100	$y = 54152x - 13\,347$	0.998	2.2	2.9
Putrescine	5–100	$y = 70574x - 78\,585$	0.997	2.2	2.6
Cadaverine	5–100	$y = 94965x - 267\,872$	0.993	1.7	2.4
Histamine	5–100	$y = 18479x - 12\,297$	0.998	3.1	4.1
Tyramine	5–100	$y = 31561x - 506\,20$	0.999	1.3	1.9
Spermidine	5–100	$y = 31754x - 1\,415.5$	0.999	1.3	2.0
Spermine	5–100	$y = 13918x - 27\,700$	0.998	1.8	2.6

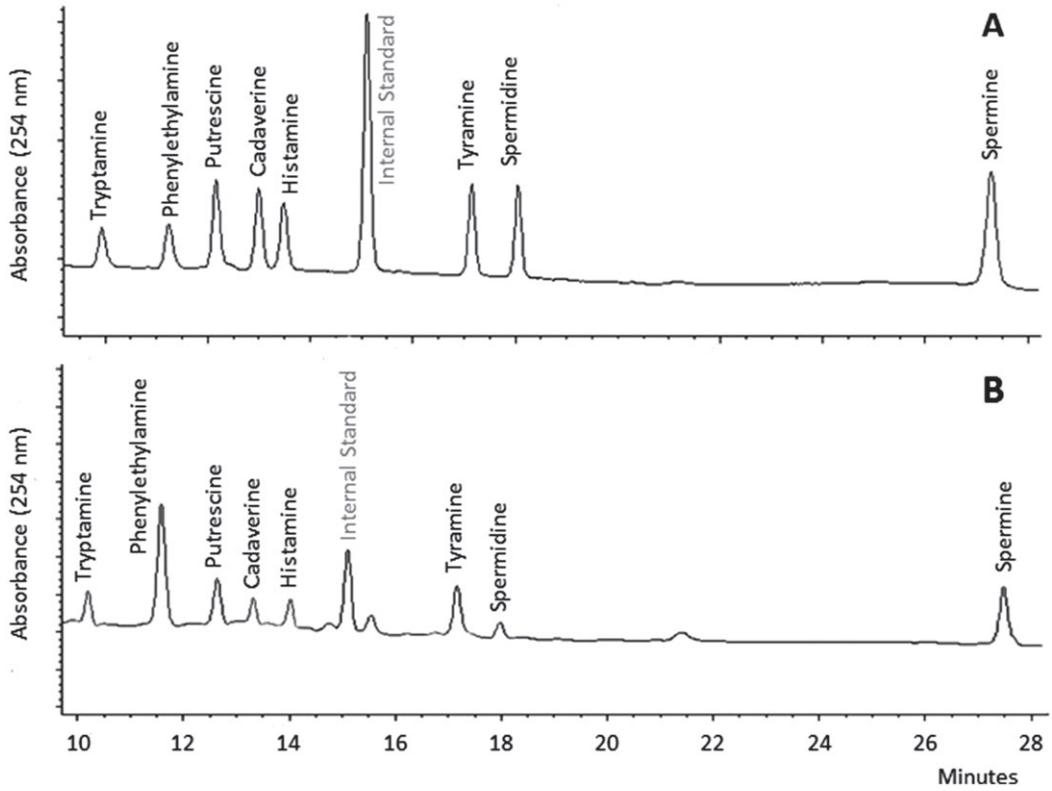

Figure 2. HPLC chromatograms relative to a standard solution (A) and a Zamorano cheese sample (B).

two supernatants were mixed and dried with nitrogen. Finally, the dry residue was redissolved in 2 mL acetonitrile and filtered with a syringe filter 0.45 µm (Thermo Scientific, Waltham, MA, USA).[20]

Chromatographic analysis was performed with a chromatographic system consisting of an Waters Alliance HPLC instrument, equipped with a Waters 2690 separation module connected to a Waters 2487 dual λ absorbance detector. Separation of analytes was carried out using a Waters Spherisorb® ODS2 analytical column (5 µm particle size, 250 mm × 4.6 mm i.d.) equipped with a Waters Spherisorb® Guard Cartridge precolumn (5 µm particle size, 10 mm × 4.6 mm i.d. and using the software package Millennium 32 Chromatography Manager™, Version 3.05, by Waters (Milford, MA, USA). The injection volume was 10 ~L. BA were separated using a gradient obtained from (A) ultrapure water and (B) acetonitrile. The elution gradient was as follows: 0% of B for 9 min, ramped at 65% for 1 min, 80% for 8 min, 85% for 5 min, 100% for 2 min (flow rate 0.8 mL min^{-1}) and 0% until the end of the run (30 min) (flow rate 0.1 mL min^{-1}).

The peaks were detected at 254 nm.[21] Figure 2 shows HPLC chromatograms of a standard solution and an extract of a Zamorano cheese sample.

Statistical analysis

Statistical handling of the data was by means of one-way analysis of variance (ANOVA), with the aim of determining the effect of ripening time, heat treatment and milk from different ewe breeds used in cheese-making on the dependent variable (BA). A least squares difference (LSD) test was applied with a confidence interval of 95% ($P < 0.05$) using the computer program Statistica, Version 6.0 (Statsoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Physicochemical analysis

Table 2 shows the changes in physicochemical parameters during ripening of Zamorano cheese. Dry matter increased significantly ($P < 0.001$) during ripening, remaining after 6 months at values that ranged between 700 and 730 g kg^{-1} cheese. Fat in dry matter content in cheeses elaborated with pasteurized milk was significantly lower ($P < 0.01$) than in those elaborated with raw milk. A higher concentration ($P < 0.01$) of protein was observed in batches made from pasteurized milk; this fact was explained by the effect of denaturing whey protein exerted by pasteurization treatment.[23] Lactose concentration decreased progressively and was virtually undetectable after 2 months of ripening; it was slightly higher ($P < 0.05$) in chesses elaborated with pasteurized milk than in those elaborated with raw milk. These results are similar to those described by other authors for this cheese variety[7] and are in accordance with the regulation of PDO Zamorano cheese.[6]

S/M ratio increased significantly ($P < 0.001$) during ripening and from 6 to 10 months was higher than that described by Thomas and Pearce[24] as optimal for enzyme activity in cheese (ranging from 4% to 6%). The a_w decreased significantly during ripening whereas pH values remained stable around 5.3–5.4. S/M ratio and pH were the variables more affected by including milk of a different ewe breed. S/M ratio was significantly lower ($P < 0.05$) and pH values significantly higher ($P < 0.05$) in cheeses elaborated with milk 100% from Churra breed than other batches of cheese. It was observed that these cheeses presented more moisture content than other batches; this greater water content, on one hand, reduced the S/M ratio and, on the other hand, improved microbial aminopeptidase activity (as can be seen in Fig. 2), which leads to a greater presence of free amino acids. These free amino acids are catabolized and many of the products liberated as a result of

Table 2. Changes in physicochemical parameters during ripening of Zamorano cheese

	Ripening time (days)								
	1	60	120	180	240	300	RT	HT	TM
Dry matter[a]	542.6 ± 19.8	638.8 ± 15.0	671.9 ± 15.2	703.0 ± 13.6	721.7 ± 13.3	736.5 ± 11.3	***	NS	NS
Fat[b]	545.2 ± 31.3	538.5 ± 24.7	527.4 ± 30.1	515.6 ± 22.1	523.0 ± 23.8	549.3 ± 30.4	*	**	NS
Protein[b]	387.5 ± 22.4	376.8 ± 14.7	374.5 ± 16.0	374.3 ± 14.2	375.5 ± 10.8	375.9 ± 19.4	*	**	NS
Lactose[b]	14.5 ± 1.9	2.8 ± 1.2	0.8 ± 0.3	ND	ND	ND	**	*	NS
S/M[c]	3.1 ± 1.3	49.0 ± 2.9	54.9 ± 5.4	64.1 ± 7.2	69.8 ± 6.0	79.0 ± 7.7	***	NS	*
pH	5.52 ± 0.13	5.35 ± 0.08	5.36 ± 0.08	5.37 ± 0.08	5.36 ± 0.09	5.38 ± 0.05	**	NS	*
a_w	0.994 ± 0.002	0.956 ± 0.003	0.944 ± 0.004	0.934 ± 0.004	0.930 ± 0.004	0.917 ± 0.007	***	NS	NS

[a] g kg^{-1} cheese;
[b] g kg^{-1} dry matter;
[c] salt/moisture ratio in g NaCl kg^{-1} H_2O.
ND, not detected; RT, ripening time; HT, heat treatment of milk; TM, type of ewe's milk; NS, non-significant differences;
*$P < 0.05$;
**$P < 0.01$;
***$P < 0.001$.

these reactions produce important changes in cheese pH, which increases.[9,25]

Proteolytic analysis

Figure 3 shows changes in nitrogen fractions during ripening of Zamorano cheese elaborated with raw and pasteurized milk, and Fig. 4 shows the changes observed in these parameters during ripening of Zamorano cheese elaborated with mixtures of milk from different ewe breeds. In both graphs we can see that this variety of cheese showed a moderate extent of proteolysis throughout ripening, with average values ranging between 200 and 220 g pH 4.4-SN kg^{-1} total nitrogen (TN) at the end of the ripening time. The depth of proteolysis was much lower, reaching values around 90 g 12% TCA-SN kg^{-1} TN at the end of ripening time. However, the most significant differences between batches were observed in nitrogenous fraction 5% PTA-SN, related to peptidase and aminopeptidase activities. Cheeses elaborated with raw milk showed average values of 22.1 g kg^{-1} TN at the end of ripening time, which were almost double ($P < 0.001$) those present in cheeses elaborated with pasteurized milk (12.5 g kg^{-1} TN). These differences were more important after 4 months of ripening and were related to changes in MRS counts (Fig. 5). In addition, higher values of pH and a_w and lower values of S/M ratio would be responsible for a greater peptidase activity ($P < 0.05$) in cheeses elaborated with milk 100% from Churra breed compared with other cheeses elaborated with mixtures of milk.

Microbiological analysis

Figure 5 shows the evolution of total lactic acid bacteria (counts on MRS agar) and Enterobacteriaceae (counts on VRBGA) during

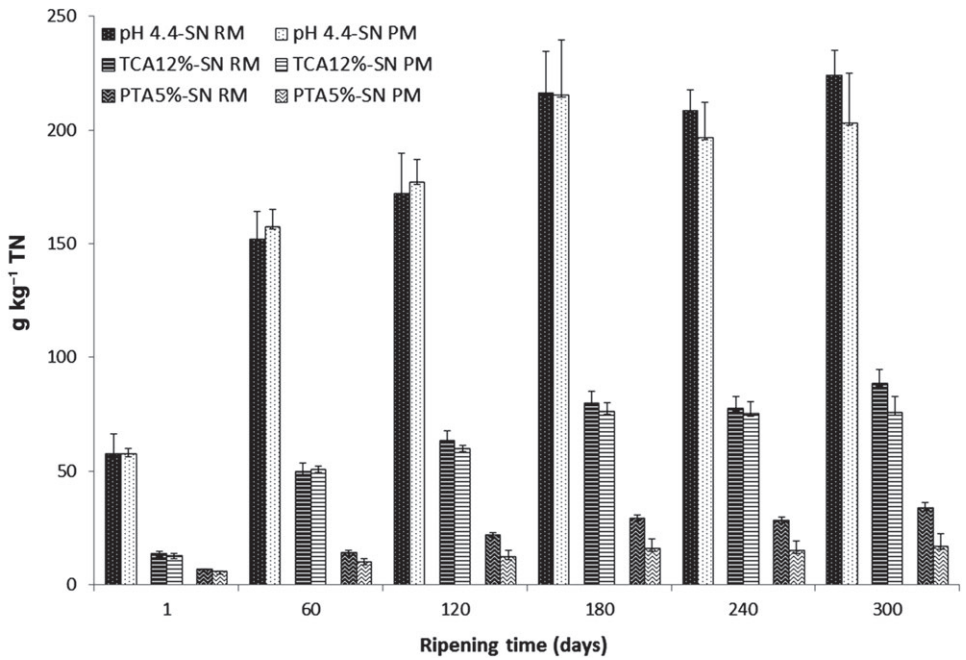

Figure 3. Changes in nitrogen soluble in water at pH 4.4 (pH 4.4-SN); nitrogen soluble in 12% trichloroacetic acid (TCA 12%-SN) and nitrogen soluble in 5% phosphotungstic acid (PTA 5%-SN) during ripening of Zamorano cheese elaborated with raw (RM) and pasteurized milk (PM).

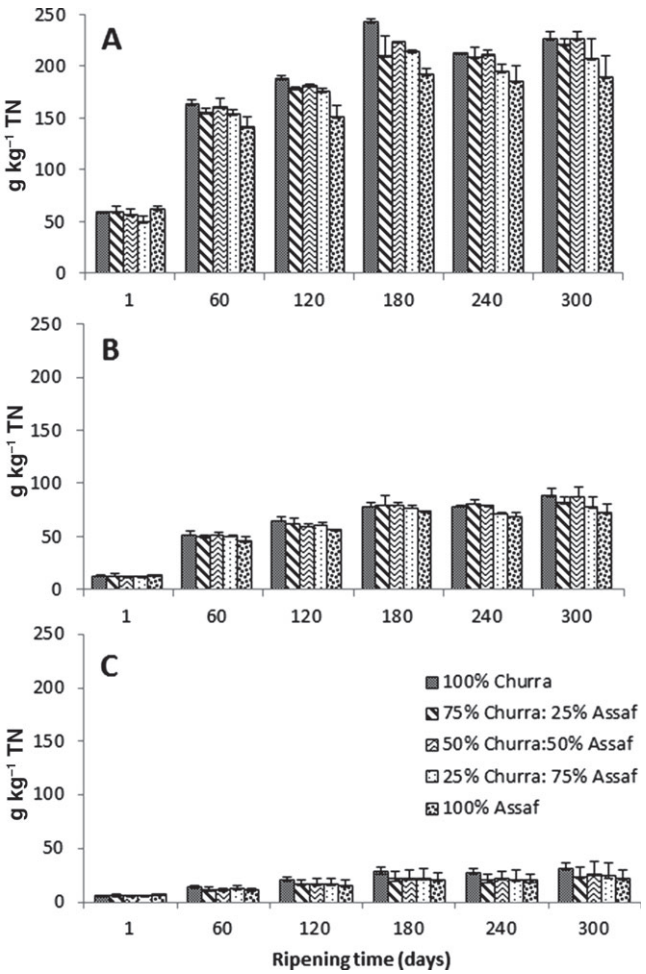

Figure 4. Changes in nitrogenous fractions in Zamorano cheese made with different mixtures of ewe's milk during ripening: (A) nitrogen soluble in water at pH 4.4; (B) nitrogen soluble in 12% trichloroacetic acid; and (C) nitrogen soluble in 5% phosphotungstic acid.

ripening of Zamorano cheese produced from raw and pasteurized milk. In general, all microbial counts were significantly reduced during ripening. However, microbial counts in cheeses made from raw milk were, in all cases, significantly higher ($P < 0.001$) than those reported for cheeses made from pasteurized milk. Zamorano cheeses made with raw milk showed counts in MRS agar around 8 log units throughout ripening, whereas those produced from pasteurized milk showed this value only in the first 2 months of ripening, decreasing between 2 and 4 log units during the remaining time of ripening. This behaviour was also described by Ferrazza *et al.*[26] in another study of Zamorano cheese made with pasteurized milk. In cheeses elaborated from pasteurized milk *Lactococcus* from starter culture proliferates very quickly in the early stages of ripening and produces a great amount of lactic acid from lactose fermentation.[26,27] After 2 months *Lactococcus* counts decrease due to acidic conditions. In cheeses elaborated from raw milk, high counts in MRS agar were observed because *Lactobacillus*, which forms part of the indigenous acid lactic bacteria, became the predominant microbiota to the end of ripening as it is adapted to acidic conditions.[26,27] Likewise, cheeses made with raw milk presented higher counts of Enterobacteriaceae and remained viable for a longer time (up to 4 or 6 months) than those produced from pasteurized milk (up to 2 months).

Figure 6 shows the average values of microbial counts on MRS and VRBGA in Zamorano cheese produced with mixtures of milk from different ewe breeds. Microbial counts in MRS were similar in all batches of Zamorano cheese, with average values around 7–8 log CFU g^{-1}. However, the batch made with 100% of milk from Churra breed presented slightly higher counts (around 1–2 log CFU g^{-1}) during all ripening. Finally, the excellent hygienic and sanitary conditions applied during the elaboration of Zamorano cheeses in this study were highlighted by the low counts for Enterobacteriaceae in all batches, which were not detected after 2 months of ripening. The average VRBGA counts observed in cheeses elaborated with 100% milk of Churra breed was significantly lower ($P < 0.05$) than other batches of cheese.

Effect of heat treatment of milk on biogenic amine content

Figure 7 shows the changes in biogenic amine content during ripening of Zamorano cheeses produced from raw and pasteurized milk.

In respect of BA analysis, phenylethylamine, putrescine, spermine and tyramine were the main BA at the end of ripening in Zamorano cheese elaborated with raw milk, whereas in those made with pasteurized milk the main BA were tyramine and spermine. During ripening, significant differences in biogenic amine content between the cheeses made with raw milk and those made with pasteurized milk were observed. The most marked differences, at an individual level, were noted in putrescine, phenylethylamine and spermine, whose concentrations at the end of ripening in Zamorano cheese produced from raw milk were from three to seven times greater than those found in cheese made from pasteurized milk. In contrast, the concentrations of tryptamine and cadaverine remained practically constant and at similar values in both types of cheese.

The total BA content in Zamorano cheese made with raw milk at the end of ripening reached 574.48 mg kg^{-1} *versus* 243.55 mg kg^{-1} in the Zamorano cheese produced from pasteurized milk. These results agree with those reported by other authors for cheeses of similar characteristics.[28–30] Nevertheless, in all these studies the final concentration of BA was much greater than what was recorded in Zamorano cheese. These differences can be related to, on one hand, the excellent hygienic conditions prevailing during Zamorano cheese-making and, on the other hand, their microbial counts, which were much lower throughout ripening than those described for other varieties.[31]

High microbial counts, especially of enterococci and enterobacteria, have been linked to higher concentrations of BA in cheese.[30] This has been related by some authors to a significant decarboxylase activity attributed to these genera.[31–33] Nonetheless, the main differences in the biogenic amine content between batches of Zamorano cheese made with raw and with pasteurized milk could be related to a greater presence of non-starter lactic acid bacteria and especially of the genus *Lactobacillus*. During ripening, environment acidification promotes the growth of lactobacilli because of a higher tolerance to acid conditions than lactococci, and the former becomes the dominant flora.[26] Fernandez-García *et al.*[34] observed that among acid lactic bacteria lactobacilli were predominant in Zamorano cheeses PDO made with raw milk. In another study of Zamorano PDO cheese made with pasteurized milk, Ferrazza *et al.*[26] reported that predominant microbiota on MRS agar was made up of lactobacilli and their counts were 2–4 log units higher than those of lactococci in the latest stages of ripening.

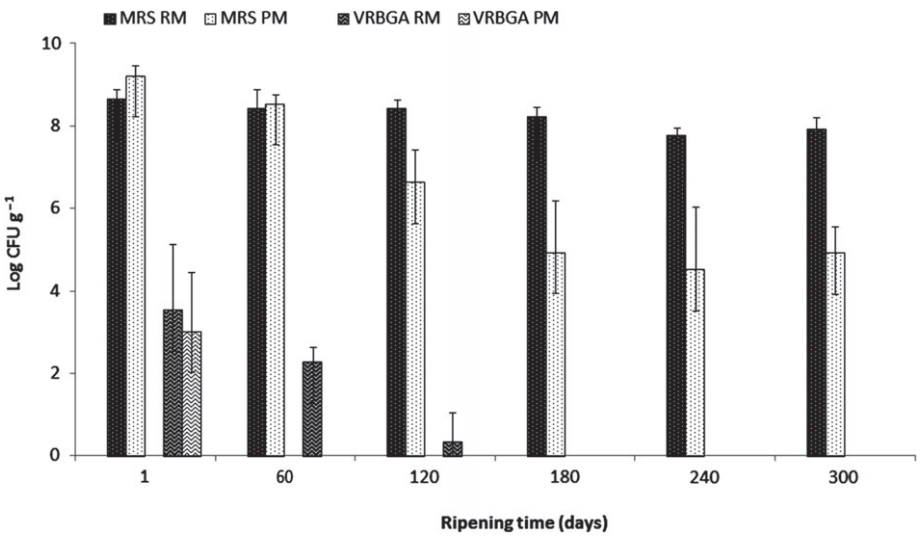

Figure 5. Changes in total lactic acid bacteria (MRS) and Enterobacteriaceae (VRBGA) during ripening of Zamorano cheese made with raw (RM) and pasteurized milk (PM).

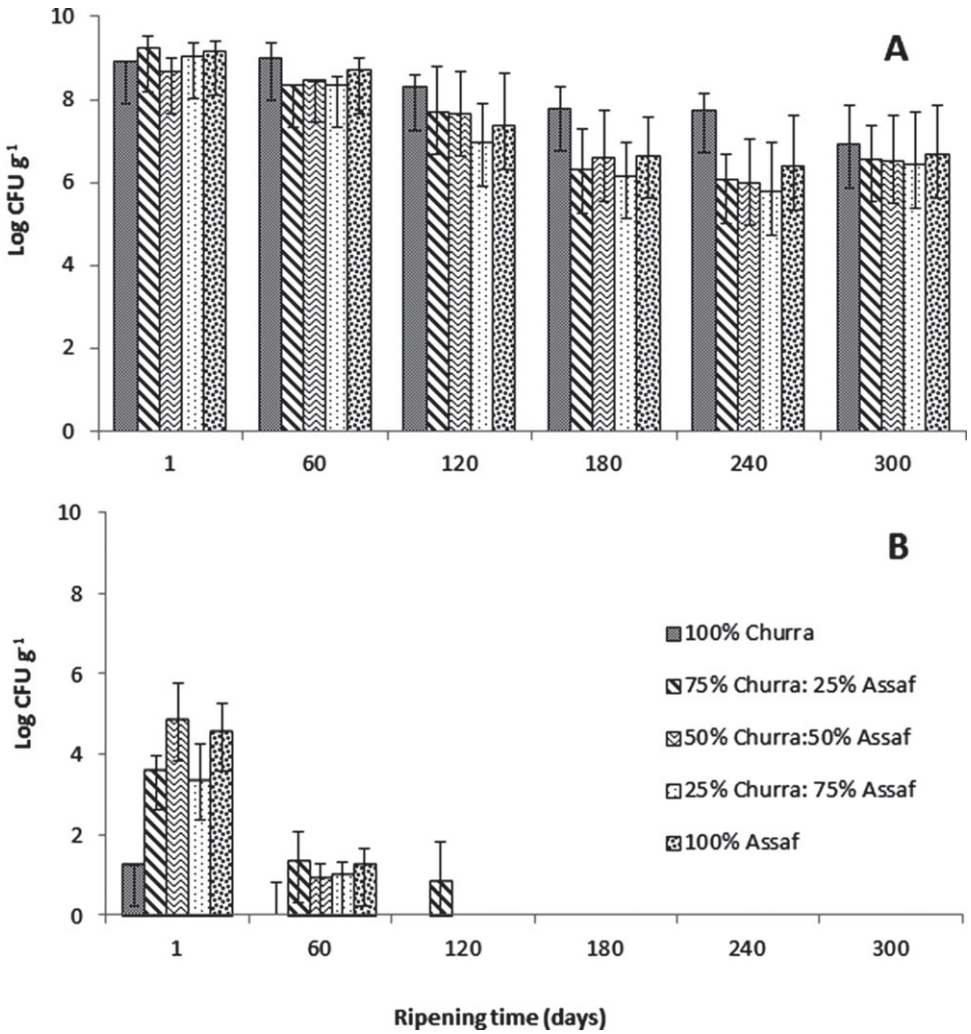

Figure 6. Changes in microbial counts in Zamorano cheese made with different mixtures of ewe's milk during ripening: (A) total lactic acid bacteria; and (B) Enterobacteriaceae.

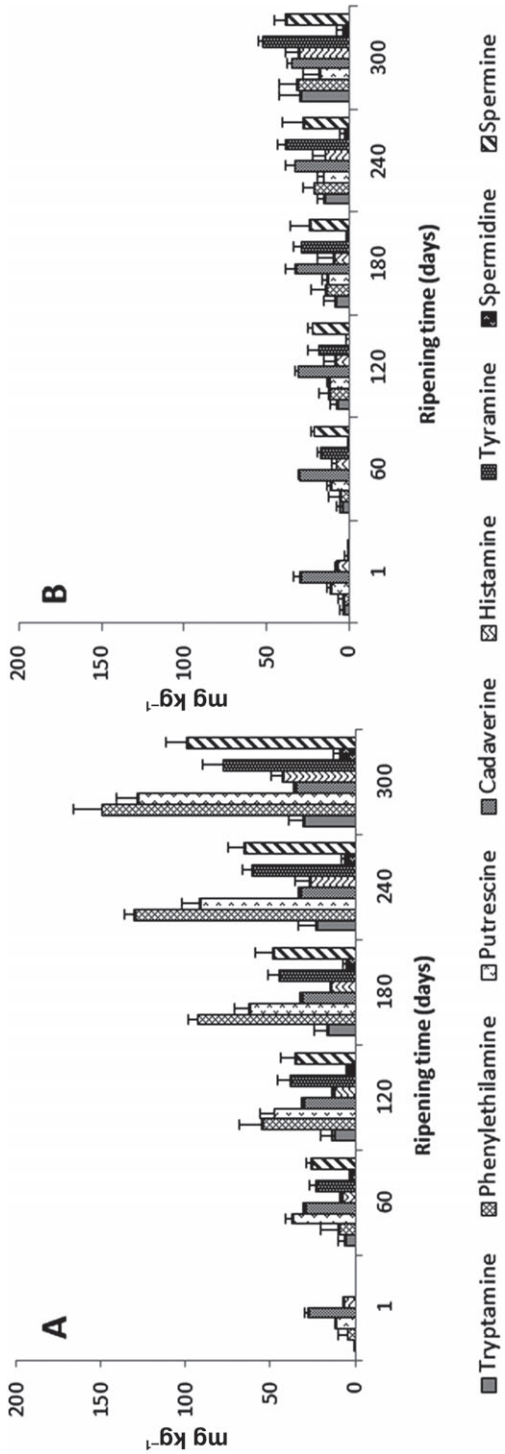

Figure 7. Changes in biogenic amine content (mg kg^{-1}) during ripening of Zamorano cheese elaborated with raw milk (A) and pasteurized milk (B).

On the other hand, it is necessary to consider the role of enterococci in the production of BA. Some authors[35–38] have associated enterococci with the formation of phenylethylamine, putrescine and tyramine. The differences observed in BA profiles in cheeses made with raw and pasteurized milk can be explained by counts of enterococci. Fernandez-García *et al.*[34] observed high counts of enterococci in Zamorano cheeses made with raw milk, whereas Ferraza *et al.*[26] described low enterococci counts in their study in Zamorano cheeses made with pasteurized milk. Moreover, a higher level of proteolysis in the cheeses made from raw milk also would explain the differences observed in BA content between cheeses made with raw and pasteurized milk. The average of PTA 5%-SN, which indicates aminopeptidase activity, during ripening of Zamorano cheeses produced from raw milk was almost double what was found in the cheeses made with pasteurized milk (Fig. 3). Furthermore, another factor of importance that would contribute to an explanation of the differences in the concentrations of BA between cheeses made with raw and with pasteurized milk would be the presence of pyridoxal phosphate; it acts as a co-factor decarboxylase activity but is inactivated by milk pasteurization, as reported Novella-Rodríguez *et al.*[39]

A striking low histamine content was observed over the course of Zamorano cheese ripening, never exceeding 40 mg kg^{-1}. This biogenic amine is one of the most problematic because it is associated with outbreaks of food poisoning and is used as an indicator of hygienic and sanitary quality of foodstuffs.[39] In view of histamine and tyramine content detected in Zamorano cheese a moderate consumption, up to a ripening time of 8 months, would not have a negative impact on the health of consumers, taking into account toxic concentrations described by other authors.[8,40] However, it is necessary to consider the specific characteristics of different individuals.

The polyamine content (spermine and spermidine) in Zamorano cheese was greater than that reported by Novella-Rodríguez *et al.*[39] and Komprda *et al.*[41] for other ripened cheeses. Spermine content was significantly higher in cheeses made with raw milk. These variations are associated with the fact that this polyamine can be formed also by an alternative metabolic pathway different from decarboxylation. Spermine is derived from putrescine and spermidine by incorporating aminopropyl groups originated from methionine.[41] The higher content of putrescine and free amino acids in cheeses elaborated with raw milk could be related to a higher counts in MRS and a higher aminopeptidase activity than in cheeses elaborated with pasteurized milk and would explain the differences in polyamine concentration between both types of cheese. Polyamines have been reported to be indispensable in various physiological/metabolic processes of cell differentiation and growth.[42]

Finally, it should be noted that the total concentration of BA detected in Zamorano cheese at the end of ripening represented approximately 40% of the maximum quantity reported by Taylor[43] as responsible for the appearance of problems of food poisoning.

Effect of type of ewe's milk on BA content

Figure 8 shows the average concentration of BA analysed in different Zamorano cheeses made with various proportions of ewe's milk from Churra and Assaf breeds. All BA showed significant differences ($P < 0.05$) between the different batches of Zamorano cheese. These were most marked in the case of phenylethylamine, putrescine, tyramine and spermine.

Cheeses produced exclusively or mostly with milk from Churra breed presented greater contents of putrescine, tyramine and

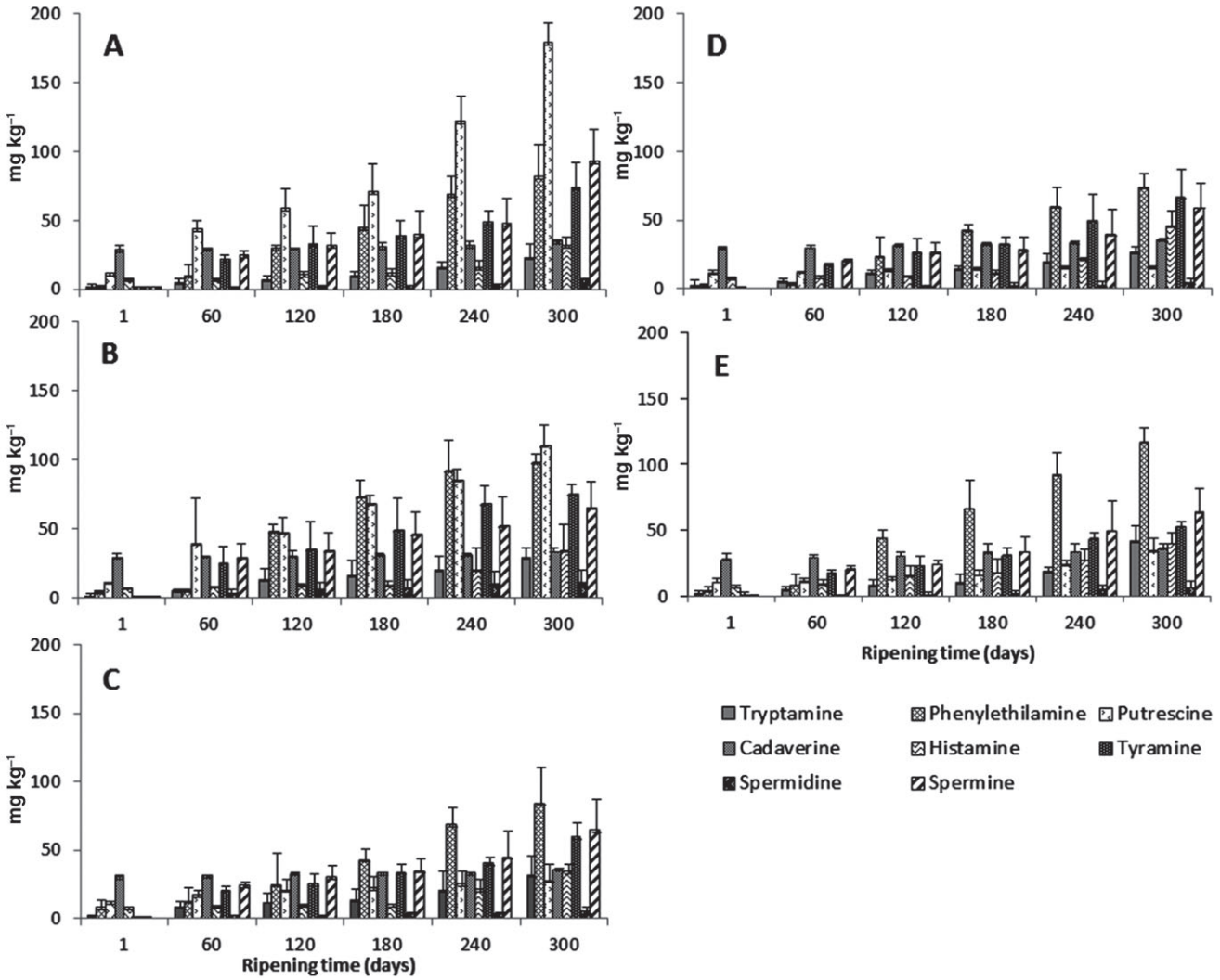

Figure 8. Changes in biogenic amine content in Zamorano cheese made with different mixtures of ewe's milk during ripening: (A) elaborated with milk 100% from Churra breed; (B) 75% from Churra and 25% from Assaf; (C) 50% from Churra and 50% from Assaf; (D) 25% from Churra and 75% from Assaf; (E) 100% from Assaf breed.

spermine. In contrast, in cheeses where milk from Assaf breed was predominant, the greatest concentrations corresponded to tryptamine, phenylethylamine and histamine. These differences in biogenic amine content between the different batches of Zamorano cheese were due not only to slight differences in microbial counts (mainly non-starter bacteria), but also and principally to the differences in proteolysis levels observed among batches. Cheeses elaborated from 100% Churra milk showed higher microbial counts on MRS than other batches of cheese by almost 1 log unit; these differences were more important after 6–8 months, the stage of ripening at which lactobacilli are, in general, the dominant microbiota. Furthermore, various studies[33,35] have described a major decarboxylase activity in strains of lactobacilli. The type of milk used seems to determine counts and diversity of this microbial group in cheese during ripening and influences the type of accumulated BA.

It has been observed that Enterobacteriaceae can produce BA *in vitro*.[44] However, it would seem that Enterobacteriaceae had no influence in establishing significant differences in BA content in Zamorano cheese made with different proportions of milk from Churra and Assaf breeds. This fact was checked because cheese batches with the highest counts for Enterobacteriaceae did not present the greatest concentrations of BA. The low impact of Enterobacteriaceae on uncooked pressed cheeses has been associated with the complexity of this system, which is determined by many factors: physical, chemical, environmental, and those arising from interrelationships between the different microbial populations that are present.[45]

Finally, extension and depth of proteolysis were significantly higher in cheeses elaborated with milk predominantly from Churra breed. As result, a greater quantity of free amino acids would be able to transform to BA by microbial decarboxylases. This fact is corroborated by PTA5%-SN values in cheese batches made from 100% Churra milk, which were almost 25% higher than in the batches elaborated from 100% Assaf milk (20.2 g kg^{-1} TN *vs.* 15.5 g kg^{-1} TN).

CONCLUSIONS

BA content in Zamorano PDO cheese increases significantly ($P < 0.01$) during ripening. Phenylethylamine, putrescine, tyramine and spermine represent around 80% of the total BA present

at the end of ripening in this cheese variety. A higher aminopeptidase activity and higher counts in MRS agar observed in cheeses elaborated with raw milk were factors that had most influence on BA content in Zamorano cheese. Heat treatment applied to the milk allowed control of the microbiota responsible for BA formation, reducing the BA content in cheeses elaborated with pasteurized milk by almost 60% . In addition, significant differences in BA content were not observed with regard to inclusion of Assaf breed in the elaboration of Zamorano cheese.

ACKNOWLEDGEMENTS

The experimental work was funded by a grant from the Castilla and León Regional Government (Junta de Castilla y León, Valladolid, Spain) and the Regulatory Council of Zamorano PDO cheese (Zamora, Spain). The authors also wish to thank Jaime Alfonso Gacho (cheese-maker) and Angel Alonso (veterinary inspector) for their collaboration.

REFERENCES

1 Önal A, A review: current analytical methods for the determination of biogenic amines in foods. *Food Chem* **103**:1475–1486 (2007).
2 Maintz L and Novak N, Histamine and histamine intolerance. *Am J Clin Nutr* **85**:1185–1196 (2007).
3 EFSA (European Food Safety Authority), Scientific opinion on risk based control of biogenic amine formation in fermented foods. *EFSA J* **9**:2393 (2011).
4 Halász A, Baráth Á, Simon-Sarkadi L and Holzapfel W, Biogenic amines and their production by microorganisms in food. *Trends Food Sci Technol* **5**:42–49 (1994).
5 Glória MB and Vale SR, Determination of biogenic amines in cheese. *J AOAC Int* **80**:1006–1012 (1997).
6 European Commission, Reglamento (CE) No. 1107/96 de la Comisión de 12 de junio de 1996 relativo al registro de las indicaciones geográficas y de las denominaciones de origen con arreglo al procedimiento establecido en el artículo 17 del Reglamento (CEE) No. 2081/92 del Consejo. *Diario Oficial de las CE* **148**:1–17 (1996).
7 Fernández D, Arenas R, Ferrazza RE, Tornadijo ME and Fresno Baro JM, Zamorano cheese, in *Handbook of Animal-Based Fermented Food and Beverage Technology*, 2nd edition, ed. by Hui YH and Özgül Evranuz E. CRC Press, Boca Raton, FL, pp. 397–416 (2012).
8 Linares DM, Río BD, Ladero V, Martínez N, Fernández M, Martín MC and Álvarez MA, Factors influencing biogenic amines accumulation in dairy products. *Front Microbiol* **3**:1–10 (2012).
9 Fox PF, *Cheese: Chemistry, Physics and Microbiology*, 2nd edition. Chapman & Hall, London (1993).
10 Novella-Rodríguez S, Veciana-Nogués MT, Roig-Sagues AX, Trujillo-Mesa AJ and Vidal-Carou MC, Influence of starter and nonstarter on the formation of biogenic amine in goat cheese during ripening. *J Dairy Sci* **85**:2471–2478 (2002).
11 International Dairy Federation, *Cheese and processed cheese: determination of the total solids content (IDF Standard 4)*. IDF, Brussels (2004).
12 International Dairy Federation, *Cheese: determination of fat content – Van Gülik method. (IDF Standard 222)*. IDF, Brussels (2008).
13 International Dairy Federation, *Milk: determination of nitrogen content. Part 1: Kjeldahl method. (IDFStandard 20–1)*. IDF, Brussels (2001).
14 Association of Official Analytical Chemists, Chloride (total) in cheese. Volhard method. 935.43 Method, in *Official Methods of Analysis of the AOAC*, 15th edition, ed. by Horwitz W. AOAC, Washington, DC (1990).
15 International Dairy Federation, *Determination of the lactose content of cheese and processed cheese products. (IDF Standard 43)*. IDF, Brussels (1967).
16 Bütikofer U, Rüegg M and Ardö Y, Determination of nitrogen fractions in cheese: evaluation of a collaborative study. *Food Sci Technol – LEB* **26**:271–275 (1993).
17 International Dairy Federation, Milk and milk products. *Preparation of test samples and dilutions for microbiological examination (IDF Standard 122B)*. IDF, Brussels (1992).
18 De Man JC, Rogosa M and Sharpe ME, A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**:130–135 (1960).
19 Mossel DAA, Mengerik WHJ and Scholts HH, Use of a modified MacConkey agar medium for the selective growth and enumeration of *Enterobacteriaceae*. *J Bacteriol* **84**:381 (1962).
20 Innocente N, Biasutti M, Padovese M and Moret S, Determination of biogenic amines in cheese using HPLC technique and direct derivatization of acid extract. *Food Chem* **101**:1285–1289 (2007).
21 Moret S, Smela D, Populin T and Conte LS, A survey on free biogenic amine content of fresh and preserved vegetables. *Food Chem* **89**:355–361 (2005).
22 Eerola S, Hinkkanen R, Lindfors E and Hirvi T, Liquid chromatographic determination of biogenic amines in dry sausages. *J AOAC Int* **76**:575–577 (1993).
23 Marshall RJ, Increasing cheese yields by high heat treatment of milk. *J Dairy Res* **53:**313–322 (1986).
24 Thomas TD and Pearce KN, Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. *NZ J Dairy Sci Technol* **16**:253–259 (1981).
25 Hemme D, Bouillanne C, Métro F and Desmazeaud MJ, Review: microbial catabolism of amino acids during cheese ripening. *Sci Aliment* **2**:113–123 (1982).
26 Ferrazza RE, Fresno JM, Ribeiro JI, Tornadijo ME and Furtado MM, Changes in the microbial flora of Zamorano cheese (P.D.O.) by accelerated ripening process. *Food Res Int* **37**:149–155 (2004).
27 Feutry F, Oneca M, Berthier F and Torre P, Biodiversity and growth dynamics of lactic acid bacteria in artisanal PDO Ossau-Iraty cheeses made from raw ewe's milk with different starters. *Food Microbiol* **29**:33–42 (2012).
28 Gaya P, Sánchez C, Nuñez M and Fernández-García E, Proteolysis during ripening of Manchego cheese made from raw or pasteurized ewes' milk: seasonal variation. *J Dairy Res* **72**:287–295 (2005).
29 Gennaro MC, Gianotti V, Marengo E, Pattono D and Turi RM, A chemometric investigation of the effect of the cheese-making process on contents of biogenic amines in a semi-hard Italian cheese (Toma). *Food Chem* **82**:545–551 (2003).
30 Schneller R, Good P and Jenney M, Influence of pasteurised milk, raw milk and different ripening cultures on biogenic amine concentrations in semi-soft cheeses during ripening. *Z Lebensm Unters Forsch* **204**:265–272 (1997).
31 Pintado AIE, Pinho O, Ferreira IMPLVO, Pintado MME, Gomes AMP and Malcata FX, Microbiological, biochemical and biogenic amine profiles of Terrincho cheese manufactured in several dairy farms. *Int Dairy J* **18**:631–640 (2008).
32 Galgano F, Suzzi G, Favati F, Caruso M, Martuscelli M, Gardini F *et al*, Biogenic amines during ripening in Semicotto Caprino cheese: role of enterococci. *Int J Food Sci Technol* **36**:153–160 (2001).
33 Leuschner RGK, Kurihara R and Hammes WP, Effect of enhanced proteolysis on formation of biogenic amines by lactobacilli during Gouda cheese ripening. *Int J Food Microbiol* **44**:15–20 (1998).
34 Fernández-García E, Carbonell M, Gaya P and Nuñez M, Evolution of the volatile components of ewes raw milk Zamorano cheese: seasonal variation. *Int Dairy J* **14**:701–711 (2004).
35 Burdychova R and Komprda T, Biogenic amine-forming microbial communities in cheese. *FEMS Microbiol Lett* **276**:149–155 (2007).
36 Ladero V, Cañedo E, Pérez M, Martín MC, Fernández M and Alvarez MA, Multiplex qPCR for the detection, quantification and identification of putrescine-producing lactic acid bacteria in dairy products. *Food Control* **27**:307–313 (2012).
37 Ladero V, Fernández M, Calles-Enríquez M, Sánchez-Llana E, Cañedo E, Martín MC *et al*, Is the production of the biogenic amines tyramine and putrescine a species-level trait in enterococci? *Food Microbiol* **30**:132–138 (2012).
38 Spizzirri UG, Restuccia D, Curcio M, Parisi OI, Iemma F and Picci N, Determination of biogenic amines in different cheese samples by LC with evaporative light scattering detector. *J Food Compos Anal* **29**:43–51 (2013).
39 Novella-Rodríguez S, Veciana-Nogués MT, Izquierdo-Pulido M and Vidal-Carou MC, Distribution of biogenic amines and polyamines in cheese. *J Food Sci* **68**:750–756 (2003).
40 Ladero V, Calles M, Fernández M and Alvarez MA, Toxicological effects of dietary biogenic amines. *Curr Nutr Food Sci* **6**:145–156 (2010).
41 Komprda T, Burdychová R, Dohnal V, Cwiková O and Sládková P, Some factors influencing biogenic amines and polyamines content in Dutch-type semi-hard cheese. *Eur Food Res Technol* **227**:29–36 (2008).
42 Löser C, Polyamines in human and animal milk. *Br J Nutr* **84**:S55–S58 (2000).

43 Taylor SL, *Histamine poisoning associated with fish, cheese and other foods. Technical document VPH/FOS/85.1*, World Health Organization, Geneva (1985).
44 Coton M, Delbés-Paus C, Irlinger F, Desmasures N, Le Fleche A, Stahl V *et al*, Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses. *Food Microbiol* **29**:88–98 (2012).
45 Delbès-Paus C, Pochet S, Helinck S, Veisseire P, Bord C, Lebecque A *et al*, Impact of Gram-negative bacteria in interaction with a complex microbial consortium on biogenic amine content and sensory characteristics of an uncooked pressed cheese. *Food Microbiol* **30**:74–82 (2012).

