Microbiological, biochemical and biogenic amine profiles of Terrincho cheese manufactured in several dairy farms

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Abstract

Terrincho is a Portuguese traditional cheese, bearing a protected denomination of origin (PDO) status, which is manufactured from raw ewes' milk and ripened for a minimum period of 30 d. The objectives of this research effort were to characterize the microbiological and biochemical profiles of this cheese, manufactured in several dairy farms during the winter cheesemaking season (December through March), and establish tentative correlations between these profiles and formation of biogenic amines. For this goal, 29 cheeses from five batches, manufactured in as many dairy farms located throughout the PDO region, were analysed. The viable numbers of the total (mesophilic) microflora, enterococci, lactococci, lactobacilli, enterobacteria, staphylococci, pseudomonads, yeasts and moulds were determined by 30 d, following classical plate counting on specific media. Free amino acid and biogenic amine contents were determined by reverse-phase high-pressure liquid chromatography. The concentration of biogenic amines correlated well with microbial viable numbers, in both qualitative and quantitative terms; significant correlations were observed between enterococci and phenylethylamine ($r = 0.868$, $p < 0.0001$), and between lactococci and cadaverine ($r = 0.646$, $p = 0.002$) and tyramine ($r = 0.868$, $p < 0.0001$). On the other hand, 220 g of Terrincho cheese would have to be consumed at a given time if the threshold of worst case risk was to be attained, which appears unrealistic for a typically single-doses meal ingredient. This study has contributed to deepen the knowledge on the microbiological and biochemical features of a unique Portuguese cheese throughout ripening, and to rationalize its safe consumption in terms of biogenic amines.

Introduction

Terrincho cheese is a traditional variety of cheese, chiefly manufactured in northeastern Portugal from raw ewes' milk—which undergoes ripening for at least 30 d. It was officially granted a protected denomination of origin (PDO) status in 1994; such a legal attribute guarantees, among other deeds, that bearing the label ''Terrincho cheese'' by the final product requires a specific geographical origin of the feedstock milk, and a specific cheesemaking process. However, as often happens with other traditional dairy products, that cheese exhibits considerable variability among dairies; besides, the intrinsic characteristics of the prevailing natural environment, compiled with such other factors as farmhouse cheesemaking patterns and dairy plant layouts influence the final sensory quality of said cheese. Furthermore, the adventitious microflora of the raw milk will eventually play a relevant role—not only in the sensory characteristics, but also in the safety hazards of that product. To date, there are almost no scientifically validated data available on the microbiological characteristics of Terrincho cheese, or on the products of proteolysis therein (e.g., peptides and free amino acids, some of which act as precursors in biogenic amine synthesis). Therefore, reliable information that will permit one to effectively address the issue of microbiological safety of Terrincho cheese is still lacking. Experimentally measurable

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parameters pertaining to the characterization of Terrincho cheese—in terms of microbiological and proteolytic profiles, would therefore backup rational efforts to improve its safety in a sustained fashion, with consequent economic benefits for the dairy farmers at large.

Common chemical indicators of cheese hygienic quality include such biogenic amines as histamine, tyramine, cadaverine, putrescine, tryptamine and 2-phenylethylamine. These compounds have been detected in many kinds of ripened cheeses, whenever a high protein content—coupled with an extensive proteolytic activity brought about by the rennet or the microbiota, provide the precursors needed for decarboxylase activity (Vale & Glória, 1998). Biogenic amines are generally recognized as a serious health hazard for humans, when present in food to significant levels. According to Taylor (1985), the threshold of risk is 1000 $mg_{\text{total amines}}\,Kg^{-1}_{\text{cheese}}$, if ingestion is associated with such potentiating co-factors as amine oxidase-inhibiting drugs or alcohol, or else if there are pre-existing gastrointestinal diseases (Stratton, Hutking, & Taylor, 1991).

The major factors that constrain the qualitative and quantitative profiles of biogenic amines in cheese are the feedstock—i.e., the availability of free amino acids produced as an outcome of proteolysis, and the process—i.e., the viability of microorganisms capable of decarboxylating free amino acids. Several extrinsic processing factors may also play an important role, viz. pH, salt concentration, water availability and redox potential (Pinho, Ferreira, Mendes, Oliveira, & Ferreira, 2001).

A number of microorganisms have been reported to be amine producers, owing to their characteristic amino acid decarboxylase system; these include the genera *Bacillus*, *Pseudomonas*, *Escherichia*, *Enterobacter*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Lactobacillus, Enterococcus, Lactococcus* and *Leuconostoc* (Suzzi & Gardini, 2003). Lactobacilli appear to be very active in production (and consequent accumulation) of histamine, tyramine and putrescine; enterococci are, in turn, considered to be notorious tyramine formers; finally, *Enterobacteriaceae* can cause cadaverine and putrescine build-up, even at low biomass densities (Bover-Cid & Holzapfel, 1999).

The objective of our research effort was to characterize the microbiological and biochemical parameters of Terrincho cheese, manufactured in several dairy farms during the winter cheesemaking season, and establish correlations between these parameters and biogenic amines present therein. The former included microorganism isolation and enumeration, whereas the latter involved assessment of acidity, pH, a_w, dry matter, total fat, total protein, soluble nitrogen fractions, free amino acids and biogenic amines. The variability of the aforementioned microbiological and biochemical parameters was assessed by univariate, bivariate and multivariate analyses (whatever appropriate)—in order to permit a better interpretation of the data generated and aid in understanding of Terrincho cheese specific dynamics.

Material and methods

Cheese manufacture and sampling

Following the advice of several experienced professionals (viz. local veterinarians, farmers, cheesemakers and marketing agents), five dairy farms (coded hereafter as B, V, T, R and M)—all located in northeastern Portugal and geographically representative of the corresponding PDO, were selected for their track record of quality and regularity of Terrincho cheese production. Five batches of Terrincho cheese—one from each dairy farm, were manufactured during the winter season (December though March) and ripened for 30 d (the shortest legal ripening period). According to the traditional method, pre-filtered plain raw ewes' milk of Churra da Terra Quente breed was heated to 35 °C, and coagulated with double-strength, artisan-produced, calf rennet at a ratio of 22 mL per 100 L of milk. The resulting curds were cut, slightly drained and placed in moulds, where they were pressed (without cooking) to help in expression of the remaining whey. The cheeses were salted upon unmoulding via soaking for 20 h in a saturated solution of NaCl, and then placed (without any packaging) in ripening chambers held at 10–12 °C and 88–89% relative humidity. From each batch, four cheeses were randomly taken for monitoring various biochemical parameters, and two cheeses were picked at random for microbiological studies (since only five cheeses were available from the R dairy, only three cheeses were in this case used for biochemical analyses, and two for microbiological analyses); a total of 29 Terrincho cheeses were thus analytically tested.

The cheeses selected for sampling were coded using the letter of the corresponding dairy (i.e., B, V, T, R and M) and a number (from 1 to 6) representing each cheese within that batch, placed in refrigerated boxes, and sent promptly to our laboratories—where they were analysed on the same day.

Microbiological analyses

The viable counts of the total (mesophilic) microflora, enterococci, lactococci, lactobacilli, enterobacteria, staphylococci, pseudomonads, yeasts and moulds were determined at 30 d, in cheeses manufactured in all dairy farms considered. For this purpose, 10 g of each sample was collected aseptically into sterile sample bags (Whirl-Pak™, Cole Parmer, Chicago, IL, USA), and homogenized with sterile 2% (w/v) sodium citrate (Merck, Darmstadt, Germany), at 45 °C and for 5 min, using a dilution factor 1:10 (w/v), in a Stomacher 400 Circulator (Seward, London, UK). Sequential decimal dilutions were prepared in sterile 0.1% (w/v) peptone water (Sigma Chemical, St. Louis, MO, USA), and plated in duplicate. Lactococci and lactobacilli were grown anaerobically (Gas-Pak anaerobic system, BBL, Cockeysville, MD, USA) on M17 Agar (Lab M, Bury, UK) and Rogosa Agar (Lab M), at 30 °C for

2 and 5 d, respectively. A solution of cycloheximide (Lab M) was added, at a level of 100 mg L^{-1}, to prevent growth of yeasts. Yeasts and moulds were grown on Potato Dextrose Agar, acidified with 10 mL of 10% (v/v) lactic acid (Merck), at 25 °C for 5 d. *Enterobacteriaceae* were determined on Violet Red Bile Glucose Agar (VRBGA), enterococci on Kanamycin Aesculine Azide Agar and staphylococci on 5% (v/v) Baird-Parker, egg-yolk tellurite agar (Lab M); all media were incubated at 37 °C, for either 24 h (former two agar media) or 48 h (latter agar medium). *Pseudomonads* were grown on Pseudomonads Agar Base (Lab M) containing 1% (v/v) glycerol, and total mesophilic bacteria were enumerated on Milk Plate Count Agar (Lab M); both media were incubated at 30 °C for 48 h. The method by Miles and Misra (1938) was used to enumerate viable bacteria on the surface of all agar plates—except in the case of VRBGA, for which the pour-plate technique was used instead. In all cheese samples, colonies with different morphologies were enumerated, isolated and stored at 4 °C as slope cultures, until further characterization was in order. Identification of the various microbial groups was confirmed, using conventional tests for physiological and biochemical characterization; these tests included Gram staining, catalase and oxidase. All analyses were performed in duplicate.

Chemical analyses

A radial slice of each cheese was taken at random, and used for chemical assays. The rind of each slice was carefully removed, and the rindless material was fully shredded. The determinations of ash, NaCl, total protein and acidity were according to standard methods (AOAC, 2000a, b, c, d). Fat content determination followed the ISO method (1975). Nitrogen content was determined according to method 20B (IDF, 1993), adapted to micro conditions (i.e. sample size of ca. one-tenth of that used for the classical Kjeldahl method—0.2 g) on a Kjeltec System II, including a Digestion System 2000 and a Distilling Unit 1002 (Tecator, Höganäs, Sweden). Dry matter was evaluated using an oven from Scaltec Instruments (Goettingen, Germany), at 100 °C. The pH of the homogeneous sample, held at room temperature, was measured using a combined pH glass electrode connected to a pH-meter MicropH 2001 (Crison, Barcelona, Spain). Water activity was determined using a dedicated water activity apparatus AW Sprint TH-500 (Novasina, Pfaffikon, Switzerland). All determinations were made in duplicate.

Proteolysis analyses

Soluble nitrogen fractions

The contents of water-soluble nitrogen (WSN), nitrogen soluble in 12% (w/v) trichloroacetic acid (TCA) and nitrogen soluble in 5% (w/v) phosphotungstic acid (PTA) were determined according to the methods described by Pinho et al. (2004). The content of WSN was expressed per unit mass of total nitrogen (TN); it will be denoted hereafter as WSN/TN, and it can be viewed as a measure of the ripening extension index. The content of nitrogen soluble in 12% (v/v) TCA was also expressed per unit mass of total nitrogen; it will be denoted hereafter as 12% TCASN/TN, and it can be viewed as a measure of the ripening depth index (Furtado & Partridge, 1988). The content of nitrogen soluble in 5% (v/v) PTA was expressed per unit mass of total nitrogen; it will be denoted hereafter as 5% PTASN/TN, and it can be viewed as a measure of the extent of secondary proteolysis. All determinations were performed in duplicate.

Free amino acids and biogenic amines

Amino acids and biogenic amines were assayed according to the method of Krause, Bockhardt, Neckermann, Henle, and Klostermeyer (1995), modified by Pinho et al. (2001). In brief, a 4-g cheese sample was suspended in 15 mL of 0.2 M aqueous perchloric acid; the mixture was homogenized in an Ultra Turrax blender (Sotel, Warsawa, Poland) for 2 min, then kept in an ultrasonic bath (Heraeus, Osterode, Germany) for 30 min, and finally centrifuged at $4000 \times g$ for 20 min. Derivatization was carried out via dabsyl chloride, at 70 °C for 15 min. The reaction was quenched by placing the vials in an ice bath for 5 min.

The high-pressure liquid chromatography (HPLC) separations were performed at 50 °C, using a 150 mm × 4 mm × 3 μm Spherisorb ODS C_{18} column (Waters, Milford, USA). Elution was carried out at a flow rate of 1 mL min^{-1}, using a volumetric gradient of solution A—9 mM aqueous sodium dihydrogenophosphate, 4% (w/v) dimethylformamide and 0.1% (w/v) triethylamine (adjusted to pH 6.55 with phosphoric acid), and solution B—80% (v/v) aqueous acetonitrile. The gradient applied comprised a step of 8% (v/v) B in A during the first 2 min, followed by four sequential linear ramps, viz.: from 8% to 20% (v/v) B in A within 5 min, from 20% to 35% (v/v) B in A within 28 min, from 35% to 50% (v/v) B in A within 10 min, and from 50% to 100% (v/v) B in A within 21 min; this upper condition was then kept for an extra 11 min, before the initial conditions were recovered within 13 min. Detection was performed by measuring absorbance at 436 nm. Quantification was carried out based on a mixture of amino acid standards: aspartic acid, glutamic acid, serine, threonine, glycine, alanine, arginine, proline, valine, methionine, isoleucine, leucine, tryptophan and phenylalanine; and biogenic amine standards: ornithine, lysine, histidine, tyrosine, ethylamine, dimethylamine, tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine, cystamine and spermine (Sigma Chemical). All determinations were performed in quadruplicate.

Statistical analyses

The averages and standard deviations were calculated for each experimental parameter, pertaining to either the microbiological or the biochemical characterization.

Descriptive statistics, analyses of variance (ANOVA, using Type III Sum of Squares), pairwise comparisons of mean values (following Tukey's test), and principal component analysis (PCA) were all performed with SPSS for Windows, v. 11.5 (SPSS, Chicago, IL, USA).

Results and discussion

Cheese sampling

Although attempts were made to make the study as representative as possible, all cheeses analysed in each dairy farm were taken from a single batch. Therefore, the standard deviation values calculated reflect within-batch, rather than within-dairy variability. This apparent shortcoming was, however, unavoidable—given the difficulties in gathering reliable material (because of limited feedstock available and great distance between farms) from all dairies, and in guaranteeing that the experimental design was effectively followed (without compromising relevant conclusions by otherwise hardly overcome logistic constraints).

On the other hand, each biochemical parameter was analysed in four different cheeses, belonging to the same batch—in attempts to generate consistent results, especially in the case of free amino acids and biogenic amines (which require extraction and derivatization steps, that add to the intrinsic variability of the experimental data). However, the fact that the samples were taken from cheeses manufactured according to a similar protocol and from the same batch of milk made it somewhat acceptable to analyse biochemical parameters from one set of cheeses and microbiological parameters from another set of cheeses (all from the same batch, and produced on the same day); again, logistic constraints forced this approach—but the reliability associated with the tentative correlation sought between these two types of data was not in risk.

Microbiological composition

The numbers of total viable counts, lactobacilli, lactococci, enterococci, staphylococci, enterobacteria, pseudomonads, yeasts and moulds in Terrincho cheeses, manufactured in the five dairy farms considered, are tabulated in Table 1. One-way ANOVA was used as a basis to determine whether there were significant differences among the mean scores of each microbial group, across the five groups of cheeses. Results showed significant differences among the dairy farms, for all microbial groups. Tukey's post-hoc test was then employed to find out whether such differences, between each pair of dairy farms, were in turn significant.

High numbers of total viable microorganisms—i.e. above 10^9 cfu g^{-1}, were measured in all experimental cheeses. Lactic acid bacteria were the major constituents of the adventitious microflora prevailing in all five dairies, with typical values in cheese amounting to ca. 10^9 cfu g^{-1}; the genera *Lactobacillus* and *Lactococcus* were detected in essentially equivalent viable numbers in all cheeses, except those from the B and M dairy farms (for which *Lactococcus* was the predominant genus). Our results for total viable microorganisms and lactic microflora are consistent with those reported by previous research groups (Macedo, Costa, & Malcata, 1996; Mimoso, Firme, & Carreira, 1990; Rua, Olivares, Romero, & Aldámiz-Echebarría, 1993), who worked with other artisanal cheeses manufactured from ewes' raw milk.

The lowest viable counts (but still above 10^8 cfu g^{-1}) of both the *Lactococcus* and *Lactobacillus* genera—which were found in cheeses from the M dairy farm, may be related to the particularly high salt content and the concomitantly lower a_w (Table 2); both these conditions are unfavourable towards their growth. In fact, negative correlations between the *Lactobacillus* ($r = -0.868$, $p < 0.0001$) and the *Lactococcus* ($r = -0.447$, $p = 0.048$) counts, and the NaCl content were observed.

Table 1
Microbiological composition of Terrincho cheeses from five dairies (B, V, T, R and M), located throughout the PDO region (mean ± S.D., corresponding to two cheeses)[a]

Microbiological viability (log/cfu g^{-1} cheese)	Dairy farm						*p*
	B	V	T	R	M	F	
Pseudomonads	6.71 ± 0.46 ab	6.80 ± 0.48 ab	6.57 ± 0.58 a	6.35 ± 0.45 a	7.61 ± 0.12 b	4.626	0.0120
Yeasts and moulds	5.01 ± 0.25 bc	6.00 ± 0.83 c	4.13 ± 0.58 ab	3.95 ± 0.10 a	5.73 ± 0.01 c	15.41	0.0001
Enterobacteria	5.81 ± 0.34 a	6.48 ± 0.13 b	6.23 ± 0.04 b	6.06 ± 0.47 ab	7.39 ± 0.09 c	20.30	0.0001
Staphylococci	4.89 ± 0.09 b	5.44 ± 0.29 c	4.96 ± 0.15 b	4.13 ± 0.23 a	4.98 ± 0.14 b	23.96	0.0001
Total aerobic counts	9.50 ± 0.22 bc	9.57 ± 0.07 c	9.23 ± 0.09 b	9.34 ± 0.17 bc	8.54 ± 0.14 a	30.63	0.0001
Lactobacilli	9.31 ± 0.02 bc	9.36 ± 0.19 c	9.08 ± 0.06 b	9.33 ± 0.07 c	8.55 ± 0.09 a	42.14	0.0001
Enterococci	6.91 ± 0.11 a	7.00 ± 0.06 ab	8.17 ± 0.02 c	6.70 ± 0.34 a	7.36 ± 0.17 b	42.86	0.0001
Lactococci	10.24 ± 0.20 c	9.42 ± 0.13 b	9.15 ± 0.00 ab	9.17 ± 0.10 ab	9.04 ± 0.09 a	60.56	0.0001

[a]Fisher's *F*-value and probability *p*-value, obtained from ANOVA, are also listed. Means in rows without common letters (a–c) are significantly different ($p < 0.05$, $n = 20$).

Enterococci were found at considerably high viable numbers—i.e., ca. 10^7 cfu g^{-1}, in all cheeses ($p<0.0001$), except those originated in the T dairy, for which their numbers surpassed 10^8 cfu g^{-1}. Such high levels of enterococci may imply an active role of these microorganisms during the whole ripening process. Furthermore, they may be implicated with the presence of histamine and other biogenic amines in the final cheese (Joosten, 1988).

The numbers of enterobacteria in the cheeses assayed were of the order of 10^6 cfu g^{-1}—except for cheeses from dairy farms B and M, for which they were below 10^6 and above 10^7 cfu g^{-1}, respectively; such relatively high numbers are possible indicators of poor microbiological quality of the raw milk, and/or improper milk-handling and manufacturing practices. Nevertheless, the aforementioned values are again within the range observed by other authors (e.g., Freitas & Malcata, 2000; Macedo et al., 1996) for similar ewes' milk cheeses. The range of viable staphylococci—i.e., 10^4–10^5 cfu g^{-1}, recorded for all cheeses analysed, has been found as well in such artisanal cheeses as La Serena (Sanchez-Rey, Poullet, Caceres, & Larriba, 1993) and Serra da Estrela (Macedo et al., 1996; Tavaria & Malcata, 1998). Numbers of pseudomonads were highest (by an extra log cycle) for cheeses from dairy plant M.

Yeasts were present at various levels among the distinct cheeses, grouped by dairy farm; the differences in numbers may be due to the distinct pH and salt concentrations found between the corresponding cheeses—although no significant correlations resulted. Occurrence of yeasts in cheeses is relevant, because they have been associated with the production of flavour compounds—as a result of their relatively strong proteolytic and lipolytic activities. However, scant information is available regarding the contribution of yeasts to synthesis of biogenic amines in foods: a histidine-decarboxylase activity was found in yeasts of the genera *Debaromyces* and *Candida* isolated from fermented meat (Montel, Masson, & Talon, 1999)—and such an activity was actually above that observed in lactic acid bacteria and staphylococci. Growth of moulds was seldom observed during incubation on PDA; the periodic washings of the cheese surface with fresh water most likely accounted for such a realization.

Chemical composition

The major bulk chemical characteristics, i.e., dry matter, protein, fat and NaCl contents, as well as the bulk physical features, i.e., a_w and pH, of the various cheeses manufactured are summarized in Table 2. In general, cheeses from all five dairy farms were similar in composition, except for ash content, which correlated directly with NaCl levels. Low variability was observed in dry matter content, and in fat-in-dry-matter content and acidity, as reflected by the non-significant *F*-values of ANOVA (Table 2). Larger variability was reported for protein-in-dry-matter content, NaCl content, water activity and pH. These latter parameters have a direct influence on the rate of proteolysis in cheese. Differences in NaCl content among the five groups of cheeses may probably be ascribed to the distinct salting procedures.

Proteolysis characteristics

Soluble nitrogen fractions

The values for the various nitrogen fractions, at 30 d of ripening, of the experimental cheeses manufactured in all dairy farms, are presented in Fig. 1. The ratio WSN/TN has been used, by a number of researchers (Furtado & Partridge, 1988; Reis et al., 2000), to follow ageing of cheese. In our case, a two-fold difference in values of WSN/TN was observed between cheeses from dairy farms B and M. The TCASN, together with the PTASN been classically used as a measure of the extent of secondary proteolysis, i.e., formation of small sized peptides (2–20 residues) and free amino acids (Furtado & Partridge, 1988). A similar trend to that for WSN/TN was observed for these two indices. In the five groups of experimental

Table 2
Chemical composition of Terrincho cheeses from five dairies (B, V, T, R and M), located throughout the PDO region (mean ± S.D., corresponding to four cheeses for B, V, T and M dairies, and to three cheeses for the R dairy)[a]

Composition (%, w/w)	Dairy farm						*p*
	B	V	T	R	M	F	
Acidity content	1.45 ± 0.03 a	1.32 ± 0.12 a	2.44 ± 0.12 a	1.15 ± 0.06 a	1.20 ± 0.03 a	1.290	0.3430
Fat in dry matter	55.20 ± 0.00 a	54.96 ± 2.47 a	53.80 ± 0.78 ab	52.26 ± 2.50 ab	50.61 ± 1.01 b	6.260	0.1100
Dry matter	45.52 ± 0.28 a	46.16 ± 0.78 ab	47.72 ± 0.91 b	44.65 ± 1.59 a	45.85 ± 0.68 ab	7.610	0.0600
Protein in dry matter	45.46 ± 0.78 a	46.95 ± 0.46 a	50.73 ± 1.07 b	46.15 ± 1.84 a	40.23 ± 1.23 c	52.40	0.0001
pH	5.17 ± 0.13 a	4.94 ± 0.04 b	4.86 ± 0.03 c	5.09 ± 0.07 bd	5.12 ± 0.08 d	77.50	0.0001
NaCl in dry matter	2.54 ± 0.20 a	2.03 ± 0.16 a	2.49 ± 0.31 a	3.52 ± 0.36 b	6.26 ± 0.32 c	139.0	0.0001
a_w	0.97 ± 0.00 a	0.97 ± 0.00 a	0.97 ± 0.00 a	0.97 ± 0.00 b	0.96 ± 0.00 c	184.0	0.0001
Ash content	3.08 ± 0.06 a	3.17 ± 0.11 a	3.39 ± 0.03 b	3.56 ± 0.11 b	5.52 ± 0.10 c	598.0	0.0001

[a]Fisher's *F*-value and probability *p*-value, obtained from ANOVA, are also listed. Means in rows without common letters (a–d) are significantly different ($p<0.05$, $n=76$).

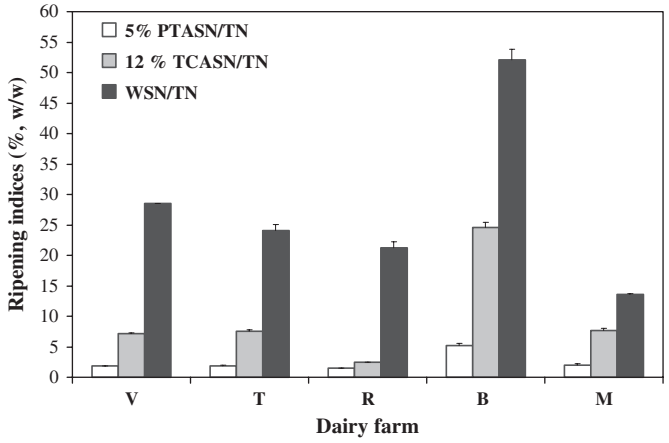

Fig. 1. Average ± S.D. of soluble nitrogen fractions (5% phosphotungstic acid-soluble nitrogen, 5% PTASN; 12% trichloroacetic acid-soluble nitrogen, 12% TCASN; and water-soluble nitrogen, WSN), per unit mass of total nitrogen (TN), for cheeses obtained from five dairy farms (V, M, T, B and R), located throughout the PDO region, at 30 d of ripening.

cheeses, cheeses from the B dairy plant exhibited the highest TCASN/TN and PTASN/TN values, followed by cheeses from V and T dairy farms (with very similar indices), and the lowest values were recorded for cheeses from R and M dairies. Correlation of these findings with the physico-chemical parameters of the various cheeses revealed that higher NaCl content promoted lower proteolytic activity in all cheeses from the M dairy (Table 2 and Fig. 1); in fact, a significant negative correlation between WSN/TN and NaCl content was observed ($r = -0.585$, $p = 0.007$). On the other hand, cheeses with lower NaCl content underwent higher proteolysis, as happened with all cheeses from the B dairy (Fig. 1). In the latter case, a higher pH was also recorded, which is probably a consequence of a higher degree of proteolysis. Both a_w and pH affect the activity of the hydrolytic enzymes responsible for casein breakdown; these include indigenous milk proteinases, rennet enzymes, and proteolytic enzymes produced by either the indigenous milk bacteria or the post-process contaminating microflora. Typically, rennet enzymes play an important role in the initial hydrolysis of casein molecules into large peptides; these subsequently serve as substrates for proteinases and peptidases released by said microflora (Sousa, Ardö, & McSweeney, 2001; Tavaria, Franco, Carballo, & Malcata, 2003).

Cheeses from the B dairy exhibited the highest viable numbers of lactococci and lactobacilli, coupled with the highest ripening indices. Significant positive correlations between viable numbers of lactococci ($r = 0.955$, $p < 0.0001$) and lactobacilli ($r = 0.565$, $p = 0.01$), and WSN/TN were observed.

Production of biogenic amines has frequently been related to the proteolytic activity of microorganisms present in cheese during manufacture and ripening. Increases in the non-protein nitrogen fractions (viz. TCASN and PTASN) often mean increased levels of free amino acids, which are precursors of biogenic amines.

Free amino acids and biogenic amines

Non-metric univariate statistics

General inspection of the experimental data was carried out for each free amino acid individually, using the minimum and maximum values observed, as well as the first quartile, the median and the third quartile. This procedure enabled construction of Figs. 2 and 3—with all results displayed simultaneously, using Box and Whisker's methodology, so as to favour comparative analyses. Strong differences between compositions of free amino acids and biogenic amines were observed; consequently, bi- and multivariate statistics were applied.

Bivariate statistics

The free amino acid contents of cheeses, from the various dairy farms, are tabulated in Table 3. One-way ANOVA was used as a basis to determine whether there were significant differences among the mean scores of each free amino acid, across the five groups of cheeses. In general, amino acid contents in cheeses within each dairy farm were distributed normally. Also, homoschedasticity was observed. The aforementioned ANOVA indicated that no significant difference was observed for tryptophan; however, significant differences were noted for all other free amino acids—hence, Tukey's post-hoc test was used to find out which of such differences were actually significant (Table 3).

As stated before, the proteolytic activity by bacteria involved in cheese ripening is quite varied, and the enzymatic action thereof is strongly influenced by quality of the raw materials and/or environmental factors. Therefore, it was not surprising to observe a considerable variation within the levels of free amino acids among the

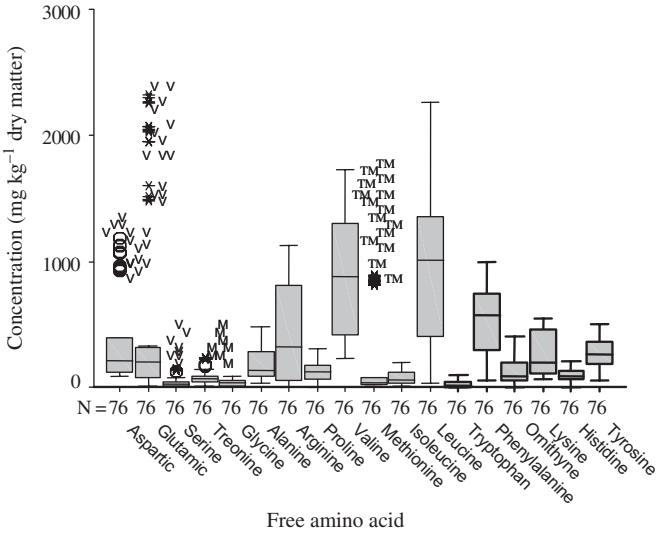

Fig. 2. Box and Whisker plot, obtained via non-metric univariate statistics, pertaining to free amino acid contents, for cheeses obtained from five dairy farms (V, M, T, B and R), located throughout the PDO region.

cheeses from the various manufacturers. Nevertheless, cheeses possessing higher PTASN/TN indices also showed higher total amino acid contents (ca. 5500 mg kg^{-1} dry matter), as expected. These final values are of the same order of magnitude as those described for other PDO ewes' milk cheeses, i.e., Idiazabal (Vicente, Ibáñez, Barcina, & Barron, 2001) and Ossau-Iraty (Izco, Torres, & Barcina, 2000).

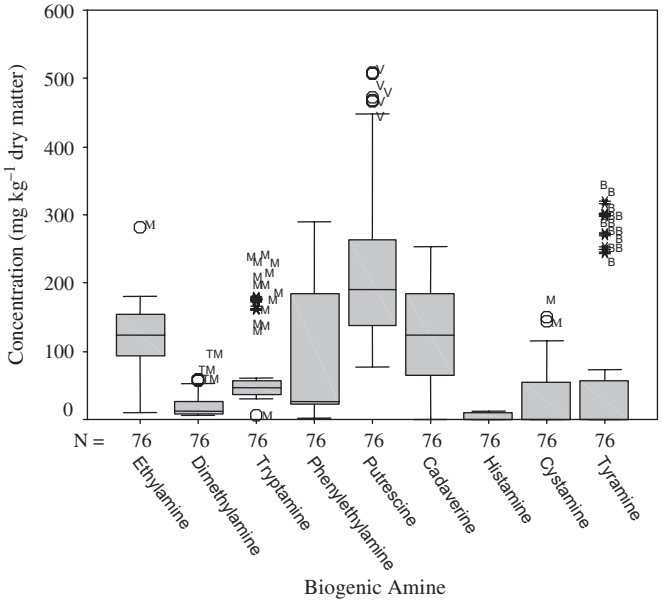

Fig. 3. Box and Whisker plot, obtained via non-metric univariate statistics, pertaining to biogenic amine contents, for cheeses obtained from five dairy farms (V, M, T, B and R), located throughout the PDO region.

Leucine, valine, aspartic acid, glutamic acid and proline were among the dominant free amino acids in the cheeses that exhibited higher free amino acid indices. Other researchers have reported amino acid profiles in ewes' milk cheeses close to those encountered herein, i.e., Manchego (Fernandez-García, Olano, Cabezudo, & Martín-Alvarez, 1993) and Gamonedo (González-de-Llano, Polo, Ramos, & Martín-Alvarez, 1991), even though different concentrations were reported for many individual free amino acids. Furthermore, it has been established (Lemieux, Puchades, & Simard, 1989) that leucine, valine, proline and glutamic acid are the most abundant free amino acids when lactococci and lactobacilli constitute the dominant flora. Our results confirm such a statement, because LAB dominate in Terrincho cheeses (especially in those from B and V dairy farms). In addition, good correlations ($r > 0.8$, $p < 0.01$) resulted between free amino acid contents and viable numbers of these microorganisms (LAB).

Concerning biogenic amine contents, results are presented in Table 4 (note that spermine was not detected at all in any of the samples analysed). The total biogenic amine content was the lowest in cheeses from R dairy, and the highest in cheeses from B dairy; cheeses from V, T and M dairies exhibited similar figures. For all cheeses, except those from dairy M, the results confirm the interrelationship between cheese proteolysis and biogenic amine production. During fermentation and ripening, the environmental factors that affect the activity of decarboxylating

Table 3
Free amino acid contents of Terrincho cheeses from five dairies (B, V, T, R and M), located throughout the PDO region (mean ± S.D., corresponding to four cheeses for B, V, T and M dairies, and to three cheeses for the R dairy)[a]

Composition (mg kg^{-1} dry matter)	Dairy farm						p
	B	V	T	R	M	F	
Tryptophan	54.8 ± 39.9 a	31.6 ± 8.4 a	20.2 ± 40.3 a	0.0 ± 0.0 a	17.5 ± 20.7 a	1.852	0.1750
Glycine	32.6 ± 3.0 a	52.2 ± 5.2 b	17.3 ± 0.6 a	6.8 ± 0.5 a	53.4 ± 31.4 b	7.066	0.0200
Arginine	964.0 ± 109.0 a	303.3 ± 65.1 bc	533.0 ± 3.7 ab	276.2 ± 2.4 bc	70.4 ± 22.0 c	11.28	0.0001
Serine	31.4 ± 4.0 a	100.5 ± 34.5 b	15.8 ± 0.8 a	14.2 ± 1.4 a	28.1 ± 15.0 a	16.14	0.0001
Threonine	49.4 ± 3.6 ab	67.8 ± 12.4 ab	78.8 ± 7.6 b	18.1 ± 3.4 a	160.6 ± 50.4 c	17.57	0.0001
Proline	118.4 ± 4.6 ab	219.9 ± 71.6 c	12.4 ± 9.0 d	68.1 ± 2.1 ad	171.2 ± 13.0 bc	22.67	0.0001
Tyrosine	264.7 ± 5.5 ab	426.7 ± 57.8 c	304.6 ± 78.0 b	191.0 ± 4.1 a	70.3 ± 6.1 d	33.58	0.0001
Histidine	68.2 ± 10.7 a	115.3 ± 22.0 b	179.6 ± 24.4 c	0.0 ± 0.0 d	89.6 ± 11.3 a	53.42	0.0001
Ornithyne	186.1 ± 20.8 a	357.9 ± 49.3 b	68.5 ± 37.6 c	90.7 ± 17.0 c	55.2 ± 4.9 c	65.01	0.0001
Isoleucine	116.6 ± 8.8 a	164.4 ± 23.5 b	49.4 ± 2.5 c	28.7 ± 1.7 cd	43.4 ± 3.6 d	111.8	0.0001
Glutamic acid	160.1 ± 42.2 a	1959.0 ± 321.0 b	290.9 ± 25.5 a	82.5 ± 6.7 a	39.9 ± 24.8 a	115.9	0.0001
Valine	1332.3 ± 1.4 a	1650.0 ± 17.7 b	890.5 ± 15.2 c	448.5 ± 47.3 d	258.0 ± 17.7 d	139.2	0.0001
Phenylalanine	64.3 ± 9.2 a	879.5 ± 78.7 b	630.6 ± 55.7 c	162.1 ± 33.1 a	406.7 ± 19.2 d	191.2	0.0001
Lysine	195.3 ± 18.9 a	444.8 ± 40.9 b	484.0 ± 31.9 b	84.7 ± 11.9 c	127.6 ± 21.1 c	166.0	0.0001
Aspartic acid	365.8 ± 24.1 a	1024.0 ± 103.0 b	122.9 ± 8.2 cd	101.3 ± 9.2 c	219.2 ± 13.4 d	230. 7	0.0001
Alanine	283.7 ± 7.7 a	446.6 ± 22.2 b	131.6 ± 14.1 c	86.7 ± 2.9 cd	59.2 ± 35.4 d	236.5	0.0001
Leucine	1264.0 ± 1.0 a	2111.0 ± 78.0 b	1015.0 ± 50.0 c	342.3 ± 73.7 d	395.5 ± 20.4 d	293.8	0.0001
Methionine	20.4 ± 1.1 a	31.6 ± 2.2 a	847.7 ± 30.7 b	31.3 ± 4.7 a	54.1 ± 15.5 a	2028	0.0001
Total amino acids	5572.0	10386.0	5697.0	2033.0	2319.0		

[a]Fisher's F-value and probability p-value, obtained from ANOVA, are also listed. Means in rows without common letters (a–d) are significantly different ($p < 0.05$, $n = 76$).

Table 4
Biogenic amine contents of Terrincho cheeses from five dairies (B, V, T, R and M), located throughout the PDO region (mean ± S.D., corresponding to four cheeses for B, V, T and M dairies, and to three cheeses for R dairy)[a]

Composition (mg kg^{-1} dry matter)	Dairy farm						p
	B	V	T	R	M	F	
Ethylamine	90.9 ± 9.6 a	143.9 ± 22.4 b	153.3 ± 12.1 b	31.0 ± 2.9 c	131.9 ± 15.9 a	39.40	0.0001
Phenylethylamine	12.9 ± 3.1 a	27.5 ± 3.3 a	237.8 ± 40.6 b	23.5 ± 1.3 a	28.3 ± 11.9 a	94.00	0.0001
Cystamine	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	88.1 ± 15.7 b	116.0	0.0001
Putrescine	247.4 ± 17.4 a	446.5 ± 31.4 b	142.5 ± 6.6 c	82.6 ± 3.6 d	191.4 ± 19.1 e	199.0	0.0001
Histamine	0.0 ± 0.0 a	0.0 ± 0.0 a	10.9 ± 0.5 b	0.0 ± 0.0 a	9.3 ± 1.2 c	337.0	0.0001
Tyramine	283.1 ± 24.2 a	0.0 ± 0.0 b	0.0 ± 0.0 b	61.4 ± 6.6 c	54.9 ± 11.2 c	346.0	0.0001
Dimethylamine	12.9 ± 0.5 a	8.1 ± 0.7 b	50.7 ± 1.7 c	7.8 ± 0.9 b	22.6 ± 2.8 d	475.0	0.0001
Tryptamine	35.6 ± 4.8 a	46.9 ± 6.3 a	47.6 ± 2.1 b	35.4 ± 1.8 a	172.9 ± 7.2 c	530.0	0.0001
Cadaverine	239.6 ± 8.7 a	84.7 ± 3.3 b	48.6 ± 3.8 c	186.3 ± 3.8 d	131.7 ± 9.0 e	558.0	0.0001
Total biogenic amines	922.0	758.0	691.0	428.0	831.0		

[a]Fisher's F-value and probability p-value, obtained from ANOVA, are also listed. Means in rows without common letters (a–e) are significantly different ($p < 0.05$, $n = 76$).

enzymes may be more important than precursor availability (Joosten, 1988; Joosten & van Boekel, 1988). Such a reasoning may explain why, despite the high concentrations of the precursor amino acid tyrosine in cheeses from V and T dairies, they do not provide evidence of tyramine in their biogenic amine inventory. The most probable factor that influences negatively tyramine production in these cheeses is their low pH. Tyramine production by *Clostridium divergens* was lower at pH 4.9 than that at 5.3, an observation apparently related to a reduced cell yield (Buncić et al., 1993).

ANOVA showed significant differences among contents of the nine biogenic amines assayed for. Putrescine was quantitatively the major biogenic amine in cheeses from V and M dairies, whereas phenylethylamine, tyramine and cadaverine were the major ones in cheeses from T, B and R dairies, respectively. High levels of putrescine and cadaverine have also been described in other types of cheeses (Suzzi & Gardini, 2003), on the other hand levels of histamine and tyramine in our work were lower than those in other traditional cheeses (Durlu-Özkaya, 2002; Innocente & d'Agostin, 2002; Roig-Sagues, Molina, & Hernandez-Herrero, 2002; Vale & Glória, 1998).

Multivariate statistics

PCA was performed using biogenic amine levels, and viable numbers of enterococci, lactococci, lactobacilli, enterobacteria and pseudomonads as variables, to reduce the dimensionality of the data and pinpoint the most important factors causing variability. The PCA results are shown in Fig. 4, and can explain 79.6% of the total variance. Dimension 1 (PC1; $K = 5.78$) accounts for 41.4% of the variance, with a Cronbach's alpha of 0.891; the negative segment of the plot for this dimension is closely related to the levels of lactococci, putrescine, cadaverine and tyramine, whereas its positive counterpart is mainly related to enterococci, ethylamine, phenylethylamine and dimethylamine. Dimension 2 (PC2; $K = 5.35$) explained 38.2% of the variance, with a

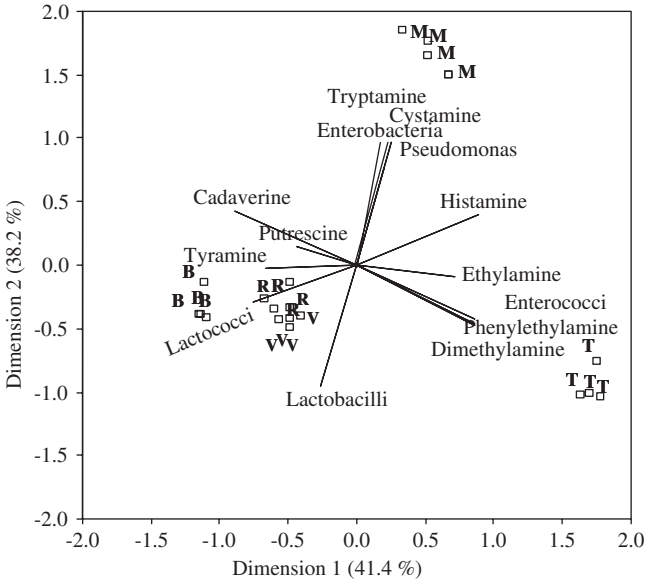

Fig. 4. Categorical principal component analysis biplot, encompassing microbial group number (by name) and biogenic amine content (by name), for cheeses obtained from five dairy farms (V, M, T, B and R), located throughout the PDO region.

Cronbach's alpha of 0.876; this dimension is positively related to levels of enterobacteria, pseudomonads, tryptamine and cystamine, and negatively related to lactobacilli.

Cheeses from B, V and R dairies presented high numbers of LAB and tyramine, whereas cheeses from M and T dairies were positioned in different segments of the biplot (Fig. 4). However, cheeses from the B dairy differed from those from V and R dairies, owing to their high numbers of viable lactococci, as well as high contents of tyramine and cadaverine. Presence of these biogenic amines is often due to metabolic activity of LAB. Significant correlations between lactococci and cadaverine ($r = 0.646$, $p = 0.002$) and tyramine ($r = 0.868$, $p < 0.0001$) further corroborated that a statement. The cheeses from the T dairy had high

levels of enterococci and phenylethylamine; as observed by Joosten (1988), Bover-Cid and Holzapfel (1999) and Montel et al. (1999), enterococci lead to accumulation of (the monoamine) 2-phenylethylamine, but are apparently not able to produce relevant amounts of (the diamines) putrescine and cadaverine. This observation was consubstantiated by significant correlations between enterococci and ethylamine ($r = 0.687$, $p = 0.001$), dimethylamine ($r = 0.951$, $p < 0.0001$), phenylethylamine ($r = 0.868$, $p < 0.0001$) and histamine ($r = 0.864$, $p < 0.0001$), but somewhat in contrast to observations by Bover-Cid and Holzapfel (1999). The distinctiveness of cheeses from the M dairy was accounted for by high levels of enterobacteria and pseudomonads, as well as tryptamine and cystamine.

Conclusions

Although the cheeses analysed were all from the same PDO region, dairy-to-dairy differences can be observed—mainly in terms of microbiological profile and/or profiles of proteolysis products and, consequently, of biogenic amines. Such differences are likely related to different degrees of hygienic quality of raw materials, and to different handling and cheesemaking practices—which consequently affect the conditions that might support growth and activity of certain groups of microorganisms, which are directly involved in proteolytic degradation and/or biogenic amine synthesis.

Our results indicate that production of biogenic amines in (raw milk) Terrincho cheese is a complex phenomenon, which depends on several variables interacting with each other. Enterococci and lactobacilli reach levels of 10^7 cfu g^{-1}, but other microbiological groups implicated in biogenic amine production (viz. enterobacteria) are also quite high in number. Significant correlations are observed between viable numbers of enterococci and phenylethylamine concentrations ($r = 0.868$, $p < 0.0001$), between lactococci and cadaverine ($r = 0.646$, $p = 0.002$) and tyramine ($r = 0.868$, $p < 0.0001$), and between enterobacteria and tryptamine ($r = 0.855$, $p < 0.0001$) and cystamine ($r = 0.830$, $p = < 0.0001$).

Furthermore, such physico-chemical parameters as high pH and low salt content seem to favour biogenic amine-positive microflora; both of these environmental factors can easily be modulated, in order to control growth of undesirable microorganisms.

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