Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 104 (2007) 693–701
Food Chemistry
www.elsevier.com/locate/foodchem

# Effects of milk high pressure homogenization on biogenic amine accumulation during ripening of ovine and bovine Italian cheeses

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Received 25 July 2006; received in revised form 26 October 2006; accepted 4 December 2006

**Abstract**

The aim of this work was to evaluate the biogenic amine (BA) content during the ripening of both bovine and ovine cheeses obtained using milk subjected to a homogenization treatment at 100 MPa before cheese-making. The data obtained were compared with those from cheeses produced by the same milks without any treatment or thermized. The results showed that both microbial ecology and BA concentrations of cheeses during ripening were significantly influenced by the type of milk used for cheese-making and by the treatment applied to the raw materials. In particular, the microbial counts found in Caciotta indicated that the high pressure homogenization (HPH) of milk significantly reduced the presence of the yeasts, *Micrococcaceae* and lactobacilli at the end of ripening. On the other hand, the HPH treatment of milk favoured the proliferation of yeasts in ovine cheese. Moreover, the ovine cheeses were characterized by a remarkably higher accumulation of BA than bovine cheeses. However, the HPH treatment of milk was able to drastically reduce the biogenic amine concentrations in both cheese typologies at the end of ripening.


*Keywords:* Cheese; Biogenic amines; High pressure homogenization; Thermization

## 1. Introduction

Biogenic amines (BA) are organic bases with aliphatic, aromatic or heterocyclic structures that can be found in several foods, in which they are mainly produced by microbial decarboxylation of amino acids, with the exception of physiological polyamines (Silla-Santos, 1996). The occurrence of BA in foods is undesirable because of their potential toxicity. In particular, tyramine, 2-phenylethylamine and histamine can cause distress, mainly due to the effects on nervous and vascular systems, which can be particularly severe in sensitive people or when the enzymes naturally involved in their detoxification (the amine oxidases) are inhibited. This repression can be due to the consumption of specific drugs (mono amine oxidase inhibitor, MAOI) or alcoholic beverages (ethanol acts as a repressor). In addition, the action of these enzymes can be depotentiated by the presence of relevant amounts of aliphatic diamines (such as putrescine and cadaverine) (Shalaby, 1996; Silla-Santos, 1996).

The BA presence in non-fermented foods generally indicates inadequate or prolonged storage. On the other hand, their presence in fermented foods could be, sometimes, unavoidable, due to the diffusion of decarboxylases among lactic acid bacteria. However, in products derived from raw materials having a high protein content (such as cheese and sausages), a great variability was observed in the total

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0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.12.017

content of BA and in its qualitative composition. These differences can be attributed to the quality of raw material, the type of product and the ripening conditions. Fermented meat products with comparable microbiological profiles may drastically differ in their BA contents, indicating that the production of such compounds depends on a complex interaction of factors (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2000; Ruiz-Capillaz & Jiménez-Colmenero, 2004; Suzzi & Gardini, 2003). The presence of relevant amounts of BA in cheeses has been recently documented (Martuscelli et al., 2005; Novella Rodrıguez, Veciana-Nogués, Izquierdo-Pulido, & Vidal-Carou, 2003; Novella-Rodriguez, Veciana-Nogues, Roig-Sagues, Trujillo-Mesa, & Vidal-Carou, 2004; Pinho et al., 2004; Valsamaki, Michaelidou, & Polychroniadou, 2000). In these studies the quantitative and qualitative accumulation of such compounds was extremely variable. However, the production of BA in cheese has been mainly attributed to the activity of non-starter microorganisms, even if an indirect role of starter LAB cannot be excluded. Generally, the main amine found was tyramine, followed by putrescine and cadaverine (Novella-Rodriguez et al., 2004; Stratton, Hutkins, & Taylor, 1991).

BA accumulation in cheese can be influenced, firstly, by the microbiological quality of raw milk, the sanitization procedures adopted, the use of starter cultures and the conditions and time of the ripening process (Novella-Rodriguez et al., 2004; Ordonez, Ibanez, Torre, & Barcina, 1997; Pinho et al., 2004). In particular, the microbial population of raw milk can influence BA presence in cheese, even when thermal treatments are applied. This is because the decarboxylase activity can be independent of the microbial cell viability and integrity (Moreno-Arribas & Lonvaud-Funel, 1999) and no data are available about the thermal sensitivities of these enzymes.

High pressure homogenization (HPH) is one of the most encouraging alternatives to traditional thermal treatment for food preservation and diversification. Its effectiveness in the deactivation of pathogenic and spoilage microorganisms in model and real systems is well documented (Kheadr, Vachon, Paquin, & Fliss, 2002; Lanciotti, Gardini, Sinigaglia, & Guerzoni, 1996; Wuytack, Diels, & Michiels, 2002).

A wide literature indicates HPH as an useful method for cell disruption and recovery of intracellular bio-products (Keshavarz Moore, Hoare, & Dunnill, 1990; Shirgaonkar & Pandit, 1998). The results obtained on cell disruption of dense microbial cultures (Shirgaonkar & Pandit, 1998) have stimulated researches on the application of HPH for food safety and shelf-life improvement. In fact, in the food industry, there is a growing interest in mild non-thermal processes, which combine an efficient microbial reduction with a maximal retention of chemical and physicochemical product properties, as well as nutritional and organoleptic characteristics of the raw materials and ingredients used.

Due to its effects on microbial cells, the application of HPH to improve safety and microbiological quality of milk and whole liquid eggs has already been proposed (Guerzoni, Vannini, Lanciotti, & Gardini, 2002; Guerzoni, Lanciotti, Westall, & Pittia, 1997). Moreover, HPH has also been proposed for large scale cell disruption and recovery of intracellular metabolites and enzymes (Bury, Jelen, & Kaláb, 2000; Geciova, Bury, & Jelen, 2002) and for bacterial bacteriophage inactivation (Moroni, Jean, Autret, & Flis, 2002). In addition to the effects on microbial cells, the HPH treatment acts on food constituents, especially proteins and enzymes, modifying their functional properties and activities (Hayes & Kelly, 2003a; Kheadr et al., 2002; Vannini, Lanciotti, Baldi, & Guerzoni, 2004). Literature reports indicate that the milk HPH treatment is a useful tool for the dairy industry, to reduce ripening span times (due to proteolysis and lipolysis enhancement) or to differentiate and innovate dairy products without detrimental effects on yields and safety, without substantial modifications of well established productive flow sheets (Lanciotti et al., 2006). These potentialities of HPH seem very interesting because innovation and cost reduction are regarded as the major sources of competitive advantages for food companies (Harmsen, Grunert, & Declerck, 2000).

To our knowledge, there are no reports on the relationships between BA content in cheese and the use of HPH treatments on milk before cheese-making. There are only few reports on the use of high hydrostatic pressure for the sanification of milk which did not show significant BA differences in cheese obtained from thermally treated and pressurized milk (Novella-Rodriguez, Veciana-Nogues, Trujillo-Mesa, & Vidal-Carou, 2002). However, the action mechanisms of this latter treatment are completely different from those of HPH (Middelberg, 1995).

The aim of this work was to evaluate the BA content during the ripening of bovine and ovine cheeses in which the milk was subjected to a homogenization treatment at 100 MPa before cheese-making. The data obtained were compared with those from cheeses produced by the same milk without any treatment or thermized.

## 2. Materials and methods

### 2.1. Cheese making

Pecorino and Caciotta cheeses were produced using ovine and bovine milk, respectively, collected from a local dairy farm. For each milk type, a total of nine batches (approximately 50 l for each batch) were produced in a cheese dairy over three consecutive days (three batches each day) with milk from the same farm. The technological and microbiological factors were kept as similar as possible for all the batches. In particular, for Caciotta cheese-making, three bovine milk batches (one for each day of production) were HPH-treated at 100 MPa, three were thermized at 65 °C for 15 s and the remaining three were not treated. The same treatments and procedures were applied to ovine milk for Pecorino cheese production.

The milk was HPH-treated, using a one-stage continual high pressure homogenizer PANDA (Niro Soavi, Parma, Italy) equipped with a PNSA valve. Milk was previously refrigerated and subjected to HPH treatment at an inlet temperature of about 5–7 °C; the temperature increase during HPH treatment was monitored at the outlet product point. The milk, having an outlet temperature not exceeding $30 \pm 2$ °C, was then collected in receiving containers and immediately heated to 37 °C. Also raw bovine and ovine milks were heated to 37 °C while thermized milk was cooled after treatment until it reached the same temperature.

The three batches of ovine treated milk were added with natural whey starter cultures (2%), composed mainly of thermophilic lactic acid bacteria (Lanciotti et al., 2006), while the three batches of bovine milk were inoculated with milk starter culture. The milk cultures were prepared by incubating raw bovine milk, previously heated at 60 °C for 30 min, at room temperature (20–25 °C) for 10 h. The pH of milk cultures, after 10 h, was $5.4 \pm 0.2$.

After the inoculation of the natural starter cultures, commercial rennet (1:10,000 – 25% pepsin:75% chymosin; Bellucci, Modena, Italy) was added to all the ovine and bovine milk batches. Coagulation occurred within 35 min. After curd cutting and resting (15 min), the curds were cooked in whey at 50–55 °C for 5 min and placed into perforated hoops (plastic cheese moulds) for 12 h at 37 °C. Then, they were salted at 20 °C in NaCl (15%) brine. After brining, Caciotta and Pecorino cheese were ripened for 27 days and 21 days, respectively, at 16 °C. Before ripening, each cheese weighed about 2.0 kg.

### 2.2. Microbiological analysis

For each cheese sample, 10 g samples, taken at the surface and in the inner part of each cheese, were placed in 90 ml sterile saline water (9 g/l) and homogenized in a stomacher (Lab-blender 80, PBI International, Milan, Italy) for 2 min. Decimal dilutions of the homogenate were made in sterile saline water and 0.1 ml of appropriate dilutions were plated onto selective media. For the yeast count, Sabouraud Dextrose Agar (Oxoid, Basingstoke, UK) was used and the plates were incubated at 28 °C for 72 h. Counts of lactobacilli were made by plating appropriate dilutions of the samples on MRS agar (Oxoid) incubated at 32 °C for 48 h in an anaerobic jar containing $H_2$ and $CO_2$ (generated by an Oxoid BR38 kit). *Micrococcaceae* and enterococci were counted by surface-plating on Baird-Parker (with added egg yolk tellurite emulsion) and Slanetz and Bartley medium incubated at 37 °C for 48 h and 44 °C for 24 h, respectively.

The microbiological analyses of cheeses were performed immediately after salting (time 0) and after a 13 and 27 day storage for Caciotta and at time 0 and after 3, 8, 10, 14 and 21 days for Pecorino. The results are means of three replicates, i.e. cheese produced from the milk subjected to the same treatment and collected on the three different days. For each replicate, two cheeses were considered.

### 2.3. Determination of biogenic amines

For the extraction, four grammes of cheese were homogenized with 15 ml of 0.2 M perchloric acid by means of an Ultraturrax macerator at medium speed. After centrifugation (10,000 rpm for 10 min at 4 °C; Avanti J-25, Beckmann & Coulter, Palo Alto, CA, USA), the sediment was again extracted with 20 ml of 0.2 M perchloric acid and centrifuged. The two supernatant fractions were combined and made to 50 ml with 0.2 M perchloric acid. Amine derivatization was performed according to Eerola, Hinkkanen, Lindfors, and Hirvi (1993). The derivatizing agent used was dansyl-chloride (Sigma–Aldrich, Gallarate, Italy), which was solubilized in acetone for HPLC (Carlo Erba Reagents, Rodano, Italy); the solution was prepared daily and used immediately; to avoid the degradation of the dansyl derivatives all the derivatized samples were protected from light and stored at −18 °C for a maximum of 7 days.

The analyses were performed with a PU-2089 Intelligent HPLC quaternary pump, Intelligent UV–VIS multiwavelength detector UV 2070 Plus (Jasco Corporation, Tokio, Japan) and a manual Rheodyne injector equipped with a 20 μl loop (Rheodyne, Rohnert Park, CA, USA). For the chromatographic separation, an Analitical Cartridge Waters Spherisorb column, 3 μ ODS-2 4.6 mm × 150 mm, coupled with Guard Cartridge Waters Spherisorb S5 ODS2 column, 4.6 × 10 mm (Waters Corporation, Milford, MA, USA), was used with the following gradient elution: 0–5 min phosphate buffer (pH 7)/acetonitrile 35:65 5–6 min water/acetonitrile 20/80, 6–15 min water/acetonitrile 10/90, 15–25 min phosphate buffer (pH 7)/acetonitrile 35:65; flow rate 0.8 ml/min.

All the analyses were performed in triplicate and the amount of each amine is expressed as mg amine/kg by reference to a calibration curve.

### 2.4. Statistical treatment of data

The results were processed by a two-way analysis of variance (ANOVA). For each stage of ripening, milk treatments (HPH, raw and thermized) were used as independent variables (factors), while microbial counts and BA content were used as dependent variables.

The Student–Newman–Keuls test was used for comparisons of sample data. Evaluations were based on a significance level of $P = 0.05$ (Statistica software 6.0 version; StatSoft Inc., Tulsa, OK).

## 3. Results

### 3.1. General

The HPH treatment resulted in a deactivation of most microbial groups comparable with thermization in both bovine and ovine milks. The initial loads of coliforms (3.30 and 3.85 log cfu/ml in ovine and bovine milk, respectively) were reduced by 2 log units after thermal and HPH

treatment. Yeasts and enterococci counts were reduced by 1 log unit after both the treatments, while lactobacilli and *Micrococcaceae* were more resistant to HPH treatment. The initial counts of lactobacilli were 3.90 and 3.69 log cfu/ml in ovine and bovine milk, respectively, and decreased to 3.30 and 3.60 log cfu/ml in HPH-treated milks. This diminution was more evident in thermized milks (2.84 and 2.30 log cfu/ml). The initial loads of *Micrococcaceae* were 3.47 and 3.77 log cfu/ml in ovine and bovine milk, respectively, and were reduced to 2.47 and 3.47 log cfu/ml in HPH-treated milks and 2.00 and 2.16 log cfu/ml in thermized milks. The differences observed for lactobacilli and *Micrococcaceae* in relation to the treatment were statistically significant ($P < 0.05$).

### 3.2. Microbiological analyses

During ripening, the samples from Caciotta and Pecorino were analyzed, with the aim to determine the presence of enterococci, lactobacilli, *Micrococcaceae* and yeasts.

The microbial ecology of cheeses during ripening was influenced by the type of milk used for cheese-making. The microbial counts found in Caciotta (Table 1) indicated that the HPH treatment significantly ($P < 0.05$) reduced the presence of yeasts (about 1 log unit) in the cheeses taken immediately after salting (time 0), while enterococci were reduced in cheeses from HPH-treated milk if compared with samples from thermized milk but not from raw milk ($P < 0.10$). *Micrococcaceae* and lactobacilli counts were not significantly affected by milk treatment.

Also, the evolution of microbial population during ripening was influenced by milk treatment. Lactobacilli were characterized by the lowest counts in cheese from HPH milk after both 13 and 27 days ($P < 0.10$). In the ripened products, *Micrococcaceae* also had lower counts in the cheeses from HPH-treated milk ($P < 0.10$). Yeast counts significantly increased in cheeses from raw and thermized milk (reaching values above 6 log cfu/g) while, in the cheese from HPH milk, the concentration was constant an did not exceed 4 log cfu/g.

The lactobacilli and *Micrococcaceae* counts in Pecorino cheese were not significantly affected by milk treatment during any of the ripening phases (Table 2). Enterococci increased by about 1.5 log cfu/g in all the samples during ripening, even if their concentration was higher in the cheese from raw milk. No significant differences were found after 21 days of ripening. Yeasts increased their concentration, especially in the late phase of ripening and reached higher counts in cheeses from HPH milk at the end of ripening ($P < 0.10$). However, significant differences were found only with the cheese obtained from thermized milk.

### 3.3. Biogenic amine content

The samples used for the determination of microbiological counts, were also analyzed for the determination of BA. The HPLC method adopted for this analysis allowed determinations of 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermine and spermidine. The results obtained are shown in Table 3 (Caciotta) and Table 4 (Pecorino).

In the samples of Caciotta, the diamines, putrescine and cadaverine, were found in higher concentrations. Putrescine was detected in low amounts (1–4 mg/kg) immediately after salting and increased during ripening to reach a concentration of about 8–10 mg/kg, without significant differences in relation to milk treatment at the end of ripening. Cadaverine was present immediately after salting in the cheeses from raw and thermized milk in concentrations fivefold higher than in cheese from HPH milk ($P < 0.05$). This behaviour was maintained during all the ripening phases. After 27 days, its concentration was 10 mg/kg in the cheese from HPH milk, while it reached 24 mg/kg and 44 mg/kg in cheeses from raw and thermized milk,

Table 1
Microbial counts in Caciotta cheese during ripening

| Ripening time (days) | Milk treatment | Microbial group (log cfu/g) | | | |
|---|---|---|---|---|---|
| | | Yeasts | Lactobacilli | Micro-staphylococci | Enterococci |
| Time 0 | HPH | $3.35^{A}$ (0.11) | 8.17 (0.69) | 6.52 (0.42) | $6.40^{A}$ (0.09) |
| | Raw | $4.25^{B}$ (0.21) | 8.86 (0.45) | 6.38 (0.06) | $6.57^{A}$ (0.43) |
| | Thermized | $4.82^{C}$ (0.21) ** | 9.12 (0.85) NS | 6.92 (0.41) NS | $7.37^{B}$ (0.43) * |
| Time 13 | HPH | $3.77^{A}$ (0.07) | $6.86^{A}$ (0.21) | 6.31 (0.47) | $6.29^{A}$ (0.22) |
| | Raw | $6.24^{B}$ (0.09) | $7.14^{A}$ (0.36) | 6.26 (0.20) | $6.40^{A}$ (0.11) |
| | Thermized | $5.98^{B}$ (0.34) ** | $7.83^{B}$ (0.33) * | 6.63 (0.16) NS | $7.23^{B}$ (0.48) * |
| Time 27 | HPH | $3.90^{A}$ (0.04) | $7.55^{A}$ (0.12) | $6.18^{A}$ (0.38) | 7.65 (0.56) |
| | Raw | $6.03^{B}$ (0.18) | $7.91^{AB}$ (0.27) | $7.57^{B}$ (0.52) | 6.89 (0.25) |
| | Thermized | $6.82^{C}$ (0.19) ** | $8.28^{B}$ (0.38) * | $7.17^{A}$B (0.30) * | 7.55 (0.13) NS |

The data reported are the means of three replicates and the standard deviation is reported within brackets. Each sampling time also shows the result of ANOVA in relation to milk treatment. NS: $P > 0.1$; *: $P < 0.1$; **: $P < 0.05$; means within the same column, for each time, without a common superscript are significantly different ($P < 0.05$).

Table 2
Microbial counts in Pecorino cheese during ripening

| Ripening time (days) | Milk treatment | Microbial group (log cfu/g) | | | |
|---|---|---|---|---|---|
| | | Yeasts | Lactobacilli | Micro-staphylococci | Enterococci |
| Time 0 | HPH | 3.72 (0.23) | 8.66 (0.15) | 4.52 (0.22) | $4.30^{A}$ (0.18) |
| | Raw | 4.04 (0.43) | 8.46 (0.61) | 4.84 (0.41) | $5.24^{B}$ (0.12) |
| | Thermized | 4.21 (0.35) | 8.65 (0.44) | 4.63 (0.25) | $4.70^{C}$ (0.13) |
| | | NS | NS | NS | * |
| Time 3 | HPH | 3.64 (0.41) | 8.75 (0.54) | 5.10 (0.39) | $4.52^{A}$ (0.23) |
| | Raw | 4.12 (0.37) | 9.16 (0.64) | 5.66 (0.20) | $5.30^{B}$ (0.32) |
| | Thermized | 4.66 (0.41) | 8.90 (0.62) | 5.18 (0.25) | $4.95^{AB}$ (0.28) |
| | | NS | NS | NS | * |
| Time 8 | HPH | 4.23 (0.33) | 8.32 (0.62) | 5.62 (0.48) | 5.27 (0.41) |
| | Raw | 4.82 (0.48) | 9.04 (0.78) | 6.06 (0.40) | 6.03 (0.67) |
| | Thermized | 5.36 (0.68) | 8.66 (0.54) | 5.45 (0.53) | 5.63 (0.16) |
| | | NS | NS | NS | NS |
| Time 10 | HPH | 4.91 (0.36) | 8.24 (0.33) | 6.38 (0.12) | 5.69 (0.57) |
| | Raw | 5.60 (0.17) | 8.90 (0.77) | 6.93 (0.62) | 6.42 (0.38) |
| | Thermized | 5.95 (0.12) | 8.65 (0.73) | 6.24 (0.32) | 6.03 (0.42) |
| | | NS | NS | NS | NS |
| Time 14 | HPH | $7.42^{A}$ (0.42) | 8.03 (0.42) | 6.78 (0.23) | $6.20^{A}$ (0.12) |
| | Raw | $6.36^{AB}$ (0.45) | 8.88 (0.42) | 7.37 (0.61) | $7.21^{B}$ (0.12) |
| | Thermized | $5.68^{B}$ (0.34) | 8.38 (0.22) | 6.75 (0.41) | $6.20^{A}$ (0.52) |
| | | * | NS | NS | * |
| Time 21 | HPH | $7.12^{A}$ (0.28) | 7.69 (0.70) | 7.15 (0.59) | 6.20 (0.63) |
| | Raw | $6.62^{A}$ (0.25) | 8.65 (0.67) | 7.51 (0.48) | 7.18 (0.53) |
| | Thermized | $5.45^{B}$ (0.41) | 8.50 (0.47) | 6.30 (0.40) | 6.40 (0.33) |
| | | * | NS | NS | NS |

The data reported are the means of three replicates and the standard deviation is reported within brackets. Each sampling time also shows the result of ANOVA in relation to milk treatment. NS: $P > 0.1$; *: $P < 0.1$; **: $P < 0.05$; means within the same column, for each time, without a common superscript are significantly different ($P < 0.05$).

Table 3
Biogenic amine accumulation in Caciotta cheese during ripening

| Ripening time (days) | Milk treatment | Biogenic amine (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2-PHE | PUT | CAD | HIS | TYR | SPM | SPMD |
| Time 0 | HPH | 0 | $2.67^{A}$ (0.97) | $3.01^{A}$ (1.48) | $0^{A}$ | $5.30^{A}$ (0.45) | 0 | 0 |
| | Raw | 0 | 4.87 B (1.40) | $17.92^{B}$ (3.22) | $0^{A}$ | $2.76^{B}$ (0.98) | 0 | 0 |
| | Thermized | 0 | $1.52^{BA}$ (0.31) | $14.42^{B}$ (2.69) | $2.73^{B}$ (1.21) | $1.43^{B}$ (0.54) | 0 | ND |
| | | NS | * | ** | ** | ** | NS | NS |
| Time 13 | HPH | $0^{A}$ | 4.45 (0.73) | $10.66^{A}$ (1.03) | $0^{A}$ | 6.95 (1.45) | 0 | $0^{A}$ |
| | Raw | $0^{A}$ | 7.74 (1.46) | $32.62^{B}$ (3.75) | $0^{A}$ | 3.54 (0.56) | 0 | $0^{A}$ |
| | Thermized | $8.70^{B}$ (1.66) | 6.70 (1.76) | $42.31^{C}$ (5.91) | $3.30^{B}$ (0.67) | 5.71 (1.78) | 0 | $5.16^{B}$ (0.24) |
| | | ** | NS | ** | ** | NS | NS | ** |
| Time 27 | HPH | $0^{A}$ | 8.33 (1.71) | $10.10^{A}$ (2.21) | 5.73 (1.29) | 9.28 (3.07) | 0 | $2.22^{A}$ (0.26) |
| | Raw | $0^{A}$ | 10.7 (1.19) | $24.9^{B}$ (1.87) | 8.37 (1.85) | 8.97 (1.05) | 0 | $2.61^{AB}$ (0.50) |
| | Thermized | $3.87^{B}$ (1.13) | 8.30 (2.73) | $44.90^{C}$ (3.50) | 7.30 (1.41) | 5.60 (1.78) | 0 | $3.40^{B}$ (0.58) |
| | | ** | NS | ** | NS | NS | NS | * |

The data reported are the means of three replicates and the standard deviation is reported within brackets. Each sampling time also shows the result of ANOVA in relation to milk treatment.
2-PHE, 2-phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPM, spermine; SPD, spermidine. NS: $P > 0.1$; *: $P < 0.1$; **: $P < 0.05$, means within the same column, for each time, without a common superscript are significantly different ($P < 0.05$). ND, not determined; 0, under detection limit.

respectively ($P < 0.05$). No significant difference can be found for tyramine at the end of ripening, and it was present in low amounts independently of the milk treatment. Also, histamine and spermidine contents were not influenced by the milk treatment at the end of ripening, while 2-phenylethylamine was found in low concentration only in the cheese from thermized milk. In general, the BA accumulation in Caciotta cheese was rather low if compared with similar products. However, as shown in Fig. 1, the total BA content at the end of ripening was significantly

Table 4
Biogenic amine accumulation in Pecorino cheese during ripening

| Ripening time (days) | Milk treatment | Biogenic amine (mg/kg) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2-PHE | PUT | CAD | HIS | TYR | SPM | SPMD |
| Time 0 | HPH | 0 | 4.58 (2.94) | $3.56^{A}$ (1.40) | 0 | $1.50^{A}$ (1.04) | 0 | $3.92^{A}$ (0.76) |
| | Raw | 0 | 7.56 (1.19) | $6.70^{A}$ (1.61) | 0 | $0^{A}$ | 0 | $12.2^{B}$ (1.33) |
| | Thermized | 0 | 11.9 (7.04) | $34.5^{B}$ (5.69) | 0 | $40.2^{B}$ (6.43) | 0 | $10.2^{B}$ (2.21) |
| | | NS | NS | ** | NS | ** | NS | * |
| Time 3 | HPH | $0^{A}$ | $13.7^{A}$ (1.32) | $20.9^{A}$ (3.30) | 3.08 (1.87) | $37.0^{A}$ (3.87) | 0 | 4.69 (1.62) |
| | Raw | $6.27^{B}$ (1.86) | $30.0^{B}$ (5.05) | $40.9^{B}$ (7.4) | 0 | $60.5^{B}$ (9.6) | 0 | 6.10 (2.03) |
| | Thermized | $0^{A}$ | $15.3^{A}$ (3.19) | $49.4^{B}$ (2.36) | 3.34 (1.34) | $54.4^{B}$ (9.50) | 0 | 7.92 (3.16) |
| | | ** | * | * | NS | * | NS | NS |
| Time 8 | HPH | 0 | 9.44 (3.41) | 22.6 (4.63) | 4.35 (1.94) | 54.8 (7.43) | 0 | 8.16 (1.24) |
| | Raw | 12.4 (3.53) | 23.1 (6.06) | 38.8 (1.55) | 0 | 65.2 (6.97) | 0 | 7.22 (1.32) |
| | Thermized | ND | ND | ND | ND | ND | ND | ND |
| | | ** | ** | NS | NS | NS | NS | NS |
| Time 10 | HPH | $0^{A}$ | $11.0^{A}$ (3.58) | $22.7^{A}$ (4.60) | $3.92^{A}$ (0.86) | $65.9^{A}$ (12.5) | 0 | $10.2^{A}$ (1.15) |
| | Raw | $18.2^{B}$ (4.18) | $18.4^{A}$ (1.77) | $36.3^{B}$ (4.09) | $0^{B}$ | $70.1^{A}$ (12.5) | 0 | $6.59^{B}$ (1.13) |
| | Thermized | $58.3^{C}$ (17.7) | $38.7^{B}$ (10.7) | $83.2^{C}$ (6.57) | $8.60^{C}$ (2.41) | $119^{B}$ (12.7) | 0 | $10.5^{A}$ (1.33) |
| | | ** | * | ** | * | * | NS | 0.013 |
| Time 14 | HPH | $0^{A}$ | $10.74^{A}$ (2.23) | $28.3^{A}$ (6.0) | $5.75^{A}$ (1.83) | $72.1^{A}$ (8.35) | 1.34 (1.29) | 13.39 (3.53) |
| | Raw | $14.7^{B}$ (2.02) | $42.52^{B}$ (4.67) | $85.9^{B}$ (11.2) | $0^{B}$ | $110^{B}$ (22.8) | 0 | 12.4 (1.80) |
| | Thermized | $95.3^{C}$ (26.7) | $60.26^{C}$ (11.52) | $164^{C}$ (13.9) | $15.69^{C}$ (2.31) | $210^{C}$ (12.5) | 0 | 13.2 (2.21) |
| | | ** | * | ** | ** | ** | NS | NS |
| Time 21 | HPH | $19.5^{A}$ (5.24) | $14.80^{A}$ (4.46) | $20.3^{A}$ (1.88) | $3.35^{A}$ (1.32) | $62.8^{A}$ (9.08) | 0 | 10.4 (1.84) |
| | Raw | $63.3^{B}$ (4.74) | $29.28^{A}$ (5.83) | $107^{B}$ (6.20) | $6.32^{B}$ (0.59) | $162^{B}$ (12.2) | 1.49 (1.16) | 9.03 (1.39) |
| | Thermized | $155^{C}$ (19.2) | $70.92^{B}$ (11.11) | $257^{C}$ (11.7) | $23.92^{C}$ (1.67) | $350^{C}$ (21.6) | 0 | 15.9 (2.38) |
| | | ** | ** | ** | ** | ** | NS | |

The data reported are the means of three replicates and the standard deviation is reported within brackets. Each sampling time also shows the result of ANOVA in relation to milk treatment.
2-PHE, 2-phenylethylamine; PUT, putrescine; CAD, cadaverine; HIS, histamine; TYR, tyramine; SPM, spermine; SPD, spermidine. NS: $P > 0.1$; *: $P < 0.1$; **: $P < 0.05$, means within the same column, for each time, without a common superscript are significantly different ($P < 0.05$). ND, not determined; 0, under detection limit.

($P < 0.05$) reduced (about 35 mg/kg) in the cheese from HPH milk in comparison with raw milk (55 mg/kg) and, especially, with thermized milk (53 mg/kg).

The BA content in Pecorino cheese (Table 4) was higher than that in Caciotta. Also in this case, great differences among the cheeses obtained from raw, thermized and HPH milk were observed. In fact, the milk HPH treatment was able to drastically limit the accumulation of these compounds in cheese. In addition, in the samples obtained from HPH milk, the BA content increased in the first 10 days of ripening and was relatively constant during the remaining period. In contrast, the cheeses from raw and thermized milk were characterized by a continuous increase of BA concentration during ripening. Tyramine and cadaverine were the most abundant BA in Pecorino. In the samples obtained from HPH milk, tyramine reached a concentration of about 65 mg/kg, but this value was more than double (160 mg/kg) and fivefold (350 mg/kg) higher in the cheese from raw and thermized milk, respectively ($P < 0.05$). Similar behaviour was shown by cadaverine, which was found at the end of ripening at concentrations of about 20 (HPH), 106 (raw) and 256 mg/kg (thermized milk) ($P < 0.05$). Also, putrescine and 2-phenylethylamine were present at the end of ripening, in particularly high amounts in thermized milk (70 mg/kg and 155 mg/kg, respectively) ($P < 0.05$). The latter aromatic amine was also present in cheese from raw milk (63 mg/kg at the end of ripening), while it was detected in low amount (20 mg/kg) in cheese from HPH milk only in the 21 day-ripened sample ($P < 0.05$). The natural polyamines were not detected (spermine) or were present at low and constant level (spermidine). Histamine never exceeded a concentration of 10 mg/kg, with the exception of the cheese obtained after milk thermization, in which it reached a level of about 23 mg/kg at the end of ripening ($P < 0.05$).

These relevant differences were reflected in the behaviour of the total BA content shown in Fig. 2. In the cheese from thermized milk, the accumulation of amines at the end of ripening was above 800 mg/kg. In the cheese from raw milk, the total BA content was markedly reduced (377 mg/kg), while it was less than 140 mg/kg in the cheeses from homogenized milk.

## 4. Discussion

The ovine cheeses were characterized by a significantly higher concentration of amines. Few literature data are available on the specific effect of the type of milk on the BA content of cheeses. However, in a recent work, Pinho et al. (2004) observed, in a Portuguese ovine

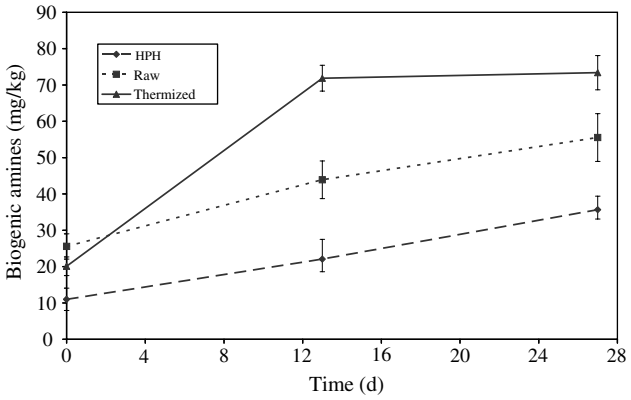


Fig. 1. Total biogenic amine contents in cheese from HPH, thermized and raw milk during the ripening of Caciotta.

cheese, a high BA content at the end of ripening (more than 900 mg/kg) and putrescine, cadaverine and tyramine were present at higher concentrations. Roig-Sagués, Molina, and Hernández-Herrero (2002) compared the tyramine and histamine contents in many Spanish cheeses, but did not find significant differences attributable to the milk used, probably because of the heterogeneity of the cheese typology (hard, semi-soft and soft) considered. An analogous heterogeneity in BA content was observed by Novella Rodríguez et al. (2003) in cheeses from bovine milk. In addition, the same authors observed a higher BA accumulation in cheeses obtained from raw milk than in similar products from pasteurized milk. Also, Schneller, Good, and Jenny (1997) found remarkably lower total BA concentrations in semi-soft cheese from thermally treated milk if compared with cheese from raw milk. By contrast, in our experimental data, the BA content was higher in cheeses from thermized milk than from raw milk, independently of the type of milk. BA accumulation is the result of the progressive action of microbial decarboxylases. It is noteworthy that the activity of these enzymes could be independent of cell integrity. Moreno-Arribas and Lonvaud-Funel (1999) demonstrated that tyrosine decarboxylase activity of *Lb. brevis* was higher in cell-free extract than in cell suspension. Therefore, it is possible that the decarboxylases produced by the microflora, directly in the milk or in the first step of cheese making, can also continue their action in cheese. This could explain the high level of aliphatic amines in the ovine cheese at the beginning of ripening, mainly attributable to the activity of Gram negative bacteria (Silla-Santos, 1996; Suzzi & Gardini, 2003). The high level of tyramine in the same cheese could be due to the activity of thermoresistant enterococci, usual contaminants of raw milk. Novella-Rodriguez et al. (2004) demonstrated that a storage, at 4 °C for 48 h, of raw goat's milk, increased the BA content of cheese with respect to the cheese obtained from fresh milk, due to the higher level of *Enterobacteriaceae* in raw milk. The balance of BA can be influenced by their demolition through the action of amino oxidases of bacterial origin. The activity of these enzymes in *Micrococcaceae* isolated from sausages has been assessed in vitro (Martuscelli, Crudele, Gardini, & Suzzi, 2002) and in vivo (Gardini, Martuscelli, Crudele, Paparella, & Suzzi, 2002; Leuschner, Heidel, & Hammes, 1998) and their activity was demonstrated in crude cell extracts.

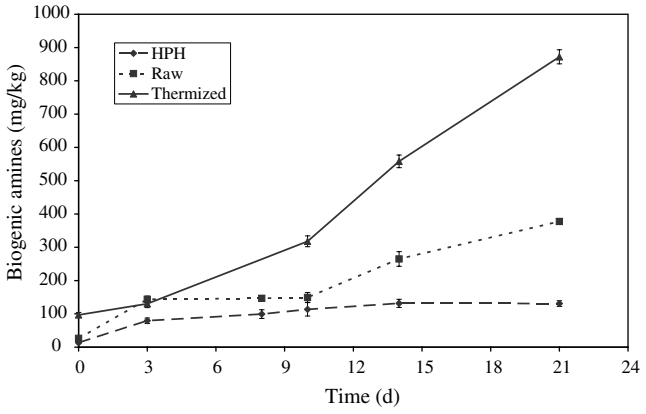


Fig. 2. Total biogenic amine contents in cheese from HPH, thermized and raw milk during the ripening of Pecorino.

Moreover, cheese is a matrix with a high protein content in which their demolition to free aminoacids (or short peptides) is guaranteed by proteases or peptidases produced by microorganisms (including starter cultures), present in milk (especially if not thermally treated) and/or in rennet. Given these activities, the availability of precursors is not a central problem in BA accumulation. The question is rather which mechanisms drive the demolition of aminoacids not necessary for cellular material synthesis toward decarboxylase or aminotransferase (and successive steps) action. Within this frame, the presence of specific aminoacids may not be indicative of specific peptidase activities but represents the results of an equilibrium between aminoacid liberation and metabolization.

The microbial data of milks used for cheese-making indicated that thermal and HPH treatments reduced the wild microflora and some quantitative differences between the two treatments could be found for lactobacilli and *Micrococcaceae*. These microbial groups were more resistant to the HPH treatment than were the others considered in this work with respect to the thermization.

The quantitative differences in the microbial counts can only partially justify the reduction of BA content in the samples obtained from HPH milk. The significant reduction of the BA presence in cheeses from HPH treatment can be mainly attributable to other causes.

In particular, we hypothesize: (i) a selection, within the different microbial groups, of species and strains with less pronounced decarboxylase aptitudes, (ii) a diminution of the decarboxylase activities, especially of those enzymes released in the medium by cell lysis or disruption before cheese-making, and (iii) an influence of the HPH treatment

on the characteristics of proteins which can affect proteolysis, the successive peptidase activity and the action of decarboxylases.

The effectiveness of HPH in the reduction of the microbial contamination level of liquid foods and cultural media is well documented (Kheadr et al., 2002; Lanciotti et al., 1996; Thiebaud, Dumay, Picart, Guiraud, & Cheftel, 2003; Wuytack et al., 2002). However, the effect on the cell load reduction depends on several factors, including the severity of treatment, the inlet temperature of the sample, the medium composition, as well as the strain and species sensitivities (Fantin et al., 1996). Consequently, in a complex microbial population, such as that of raw milk, the HPH treatment affects both the initial cell load and the qualitative initial composition of the microbiota. In addition to the modifications of the microbiota of the raw material, the HPH treatment of milk is reported to induce significant changes of the microbial populations able to grow in the product during the ripening or storage period (Guerzoni et al., 1999; Lanciotti et al., 2004, Lanciotti et al., 2006; Lanciotti, Vannini, Pittia, & Guerzoni, 2004). In Crescenza cheese, the HPH treatment of milk negatively influenced the growth of yeasts and pseudomonads and, positively, the growth of lactobacilli. In addition, the equilibrium within the different microbial groups changed: among yeasts, oxidative species prevailed whereas the pseudomonads grown after HPH treatment had a lower potential in the production of bitter peptides (Lanciotti, Vannini, et al., 2004). Moreover, Guerzoni et al. (1999) evidenced the capacity of HPH treatment to induce a significant shift in the LAB population of goat cheese during ripening, favouring heterolactic species.

According to the literature data, HPH can induce both enzyme activation and inactivation (Fantin et al., 1996; Hayes & Kelly, 2003b). In fact, since the activity of these molecules is due to their three-dimensional configuration, small changes regarding the active sites can induce an increase or decrease in their activity. Moreover, it has been reported for proteins that the large supramolecular structure is disrupted under pressure, allowing the components to move freely and become independent of the original structure. When the pressure instantaneously decreases, the original structure is not reformed (Payens & Heremans, 1969). Hayes and Kelly (2003b) attributed the pH decrease of HPH-treated (50–200 MPa) whole milk to the action of the indigenous lipoprotein lipase, apparently not inactivated, combined with reduced fat globule size, responsible for a greater potential for lipolysis. On the other hand Hayes and Kelly (2003a) evidenced a reduction of plasmin activity in HPH-treated milk, attributed to the disruption of casein micelles, having a protective action on the enzyme.

Recent works have demonstrated that HPH can affect protein or polysaccharide denaturation, aggregation and microparticle formation. Moreover, this technology can be applied to the formation of complexes between proteins, fat globules and polysaccharides. The effects on these constituents, in addition to a modification of their functional properties and to changes in food microstructure, can also affect the activity of the enzymes and the susceptibility of the macromolecules present in the system to the enzymatic attack (Wuytack et al., 2002).

## 5. Conclusions

In this work, cheeses obtained from ovine milk always had a higher BA content than had cheeses from bovine milk, independently of the treatment applied to milk. The HPH treatment significantly reduced BA accumulation, in both Caciotta and Pecorino cheeses. Surprisingly, the higher BA contents were found in the thermized samples. It is hypothizable that the mild thermal treatment applied selected a decarboxylating microbial population, which dominated during cheese-making and, possibly during ripening.

The quali- quantitative modifications of microbiota and the changes induced in the macromolecules by the HPH treatment caused a drastic reduction of BA accumulation in cheese. Consequently, HPH, which is a promising treatment for production of cheese because of the higher yield and the shortening of ripening time induced, could also be a useful tool for reducing the accumulation of undesirable compounds, such as BA.

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