



High content of biogenic amines in Pecorino cheeses

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ABSTRACT

Pecorino refers to Italian cheeses made exclusively from raw or pasteurized ewes' milk, characterized by a high content of fat matter and it is mainly produced in the Middle and South of Italy by traditional procedures. The autochthonous microbiota plays an important role in the organoleptic traits of Pecorino cheese and it can influence biogenic amines (BA) content.

The aim of this study was to characterize from microbiological and chemical point of view 12 randomly purchased commercial cheeses produced in Abruzzo region. Moreover, the BA content and the bacteria showing a decarboxylating activity were detected. For this purpose, a real-time quantitative PCR (qPCR) was applied to evaluate histamine and tyramine-producers.

The samples were well differentiated for microbial groups composition, such as aerobic mesophilic bacteria, *Enterobacteriaceae*, coagulase-negative staphylococci, yeasts, enterococci, mesophilic and thermophilic lactobacilli. Pathogens such as *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 were absent in all samples.

In most samples the content of BA resulted to be high, with prevalence of histamine and tyramine. In particular, total BA content reached 5861 mg/kg in Pecorino di Fossa cheese. The qPCR method resulted to be very useful to understand the role of autochthonous Pecorino cheese microbiota on BA accumulation in many different products. In fact, since the ability of microorganisms to decarboxylate amino-acids is highly variable being in most cases strain-specific, the detection of bacteria possessing this activity is important to estimate the risk of BA cheese content.

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1. Introduction

Cheesemaking is one of the major industries worldwide, and great part is still practiced on relatively small scale which accounts for the rich diversity of cheese available (Fox and McSweeney, 2004). The growing interest in artisanal cheeses is partly due to the uniqueness of such products, in which specialized microorganisms can grow and contribute to their organoleptic and qualitative characteristics. However, these dairy products are often manufactured under poor or uncontrolled hygiene conditions; in addition, they are produced following different protocols, which can vary from one cheesemaker to another. Many cheesemakers use raw milk considering it essential for good and stronger flavor than pasteurized milk, primarily due to greater proteolysis and lipolysis by the raw milk microbiota in the cheese (Buffa et al., 2001). This microbiota plays a major role in the development of the organoleptic characteristics of cheeses but it can also be

responsible for the accumulation of undesirable substances, such as biogenic amines (BA). The presence of these compounds is often unavoidable in fermented foods (Ladero et al., 2010a; Suzzi and Gardini, 2003). Nevertheless, their presence can cause several problems for susceptible consumers, such as nausea, respiratory distress, hot flushes, sweating, heart palpitation, headache, bright red rash, oral burning, hyper- or hypo-tension, whose intensity is dependent on quantitative and qualitative differences (Ladero et al., 2010a; Stratton et al., 1991). Cheese is among the most commonly implicated foods associated with histamine poisoning and tyramine toxicity (EFSA Panel on Biological Hazards-BIOHAZ, 2011). In the Netherlands, United States and France several cases of BA poisoning have been described. Outbreaks involved Swiss cheese containing more than 100 mg histamine/100 g cheese. Low levels of BA could have negative health effects in adults with histamine intolerance or those being administered monoamine oxidase inhibitors (MAOI) (indirect sympathomimetic drugs). Moreover, cheeses with high content of BA could be related to pediatric and adolescent migraine (Komprda et al., 2007; Millichap and Yee, 2003). In particular, close relation between consumption of tyramine-rich foods, such as cheese, migraine crisis has been found,

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and the toxic effects have been named as *cheese-reaction* (Coutts et al., 1986; Hanington, 1967).

In cheese the most important BA are histamine, tyramine, putrescine and cadaverine, that originate from specific aminoacid decarboxylase activities of cheese microbiota. In fact, many different genera and species of Gram negative and positive bacteria possess the ability to produce BA (Linares et al., 2012).

In addition, the BA accumulation is related not only to the presence of BA-producing microorganisms, but also to several variables such as their proteolytic and decarboxylase activity, starter cultures, pasteurization, physico-chemical factors, ripening time, ripening and storage temperature, etc. (Buňková et al., 2010; Burdychova and Komprda, 2007; Fernández et al., 2007; Gardini et al., 2001; Ladero et al., 2010a; La Gioia et al., 2011; Naila et al., 2010; Martuscelli et al., 2005; Wolken et al., 2006). It is well known that the decarboxylase activity of microorganisms is highly variable, being in most cases strain-specific, so the detection of these bacteria is important not only to estimate the risk of BA food content but also to prevent their accumulation in fermented foods. In this context the application of quantitative real-time PCR (qPCR) could be useful because it allows the rapid detection of producers and the quantification of genes and gene expression (Martinez et al., 2011; Postollec et al., 2011). Different qPCR assays have been developed to detect and quantify Gram positive bacteria able to produce BA in different food matrices (Fernández et al., 2006; Ladero et al., 2010b; Lucas et al., 2008; Nannelli et al., 2008; Torriani et al., 2008). Recently, this method has been applied to detect histamine producing Gram negative bacteria in fish (Bjornsdottir-Butler et al., 2011; Martinez et al., 2011).

Due to the impact of BA on human health and food safety, monitoring their levels in foodstuffs is still gaining importance (Önal, 2007). In Italy there is a large production of Pecorino cheeses (~62,000 t of production in 2010) of different varieties, which have typical characters and originate from a delimited geographical area (Coda et al., 2006; Pirisi et al., 2011). The name Pecorino is commonly given to Italian cheeses made exclusively from pure ewes' milk and it has in most cases a Protected Designation of Origin or PDO status. Generally this type of cheese is produced in the Middle and South of Italy by a traditional procedure, characterized by a different ripening time ranging from 8 to 12 months (Di Cagno et al., 2003).

Pecorino di Farindola and Pecorino Abruzzese are traditional varieties of cheese made from ewes' milk, produced in the Abruzzo region, on the east side of the Gran Sasso mountain (National Park of Gran Sasso, Italy). These two Pecorino cheeses show unique organoleptic characteristics deriving from the environmental conditions and manufacturing practices already existing in the production area (Chaves-López et al., 2006; Martuscelli et al., 2005; Schirone et al., 2011; Serio et al., 2007). The presence of relevant amounts of BA in cheeses has been recently documented for different types of Pecorino cheeses (Bavazzano et al., 2011; Del Signore and Di Giacomo, 2008; Forzale et al., 2011; Lanciotti et al., 2007; Martuscelli et al., 2005; Mascaro et al., 2010; Mercogliano et al., 2010; Schirone et al., 2011). In these studies the quantitative and qualitative accumulation of such compounds was extremely variable (Schirone et al., 2012). In ten batches of Pecorino di Farindola (Schirone et al., 2011) tyramine resulted to be the BA present at the highest concentrations in all examined cheeses, representing in six samples more than 40% of the total amines. High values of this BA have been detected also in Pecorino Abruzzese (Martuscelli et al., 2005). The relevant incidence of tyramine in other cheeses manufactured from raw ewes' milk has been reported (Ladero et al., 2010b; Martuscelli et al., 2005; Pintado et al., 2008; Roig-Sagués et al., 2002).

In the present work, the histamine and tyramine content and histamine and tyramine producing bacteria of different commercial cheeses were determined by HPLC and qPCR, respectively.

2. Materials and methods

2.1. Samples of Abruzzo cheeses

Twelve randomly purchased commercial cheeses produced in Abruzzo region (two from each dairy farm), were analyzed (Table 1). These cheeses are produced in the local farms of Abruzzo region according to the traditional cheese making procedures, which did not include the use of starter cultures.

2.2. Microbiological analyses

Samples (10 g) of cheese were diluted in 90 ml of a sodium citrate (2% w/v) solution and homogenized with a Stomacher Lab-Blender 400 (Steward Medical, London, UK) for 1 min. Serial dilutions in sterile peptone water (0.1% w/v) were plated in duplicate on selective media to enumerate the following microorganisms: aerobic mesophilic bacteria on Plate Count Agar (PCA; Oxoid, Milan, Italy) at 30 °C for 48 h; mesophilic and thermophilic lactobacilli on MRS agar (Oxoid), acidified to pH 5.4 with acetic acid, at 30 °C and 45 °C, respectively for 48 h in anaerobic conditions using the Gas-Pack anaerobic system (AnaeroGen; Oxoid); coagulase-negative staphylococci (CNS) on Baird-Parker agar with egg yolk tellurite enrichment (Oxoid) at 37 °C for 24 h; enterococci on Slanetz-Bartley agar (Oxoid) at 37 °C for 48 h; *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBGA; Oxoid) at 37 °C for 24 h. Yeasts were grown on Yeast Peptone Dextrose Agar (YPD; 1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar; Oxoid) supplemented with chloramphenicol (150 mg/l) at 25 °C for 3–5 days. The presence of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 was determined according to standard methods (Association Française de Normalisation, 1997; International Organization for Standardization, 1998, 2002).

2.3. 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 and total protein were carried out according to standard methods (AOAC, 2000a,b). Moisture was evaluated using an oven from Wtb

Table 1
Main characteristics of cheese samples used in the study.

Product	Sample code	Type of milk	Ripening	
			Months	Temperatures (°C)
Canestrato di Castel del Monte	PA2	Sheep, pasteurized, whole	8	14–15
Pecorino di Fossa	PA3	Mix of cow and sheep, raw, whole	3	12–14
Pecorino sotto fieno	PA4	Sheep, pasteurized, whole	6	15
Pecorino di grotta	PA5	Mix of cow and sheep, pasteurized, whole	4	14–15
Pecorino abruzzese sott'olio	PA6	Mix of cow and sheep, raw, whole	8	14–15
Pecorino di Atri or Abruzzese	PA7	Sheep, pasteurized, whole	5	10–16
Pecorino d'Abruzzo	RT0	Sheep, raw, whole	10	10–14
Pecorino d'Abruzzo	RT1	Sheep, raw, whole	3	10–14
Pecorino d'Abruzzo	RT2	Sheep, raw, whole	3	10–14
Pecorino d'Abruzzo	RT3	Sheep, raw, whole	3	10–14
Pecorino d'Abruzzo	RT4	Sheep, raw, whole	3	10–14
Pecorino di Farindola	MR	Sheep, raw, whole	3	14–15

Binder (Tuttlingen, Germany), at 100 °C. Total lipids were extracted according to the method of Folch et al. (1957). pH measurement was carried out on a sample (10 g) of cheese dispersed in 10 ml of deionized water using a pH meter MP 220 (Mettler, Toledo, Novate Milanese, Italy); water activity (a_w) was measured by an Aqualab instrument (Series 3, Decagon Devices, Inc., Pullman, Washington, USA). All determinations were performed in duplicate.

2.4. Biogenic amine determination

Determination of BA was carried out by acid extraction and derivatization with dansyl chloride using the methods of Eerola et al. (1993) and Moret and Conte (1996), as reported by Martuscelli et al. (2005).

2.5. HPLC analysis

The chromatographic system consisted of an HPLC Waters Alliance (Waters SpA, Vimodrone, Italy), equipped with a Waters 2695 separation module connected to a Waters 2996 photodiode array detector. The separation of the analytes was carried out using a Waters Spherisorb C18 S30DS-2 column (3 µm particle size, 150 mm × 4.6 mm I.D.) equipped with a Waters Spherisorb S50DS2 guard column. The injection volume was 10 µl. Separation conditions were adapted from the method reported by Martuscelli et al. (2005). Biogenic amines were separated using a linear gradient obtained from acetonitrile (A) and ultrapure water (B). The elution gradient was as follows: the initial mobile phase contained 57% A and these conditions were maintained for 5 min; this percentage was linearly increased up to 80% in 4 min and, after that, the content of A increased from 80% to 90% in 1 min, maintaining these conditions for 5 min. After this period, conditions returned to initial composition. A column reequilibration step with 57% A for 5 min was carried out prior to next injection. The flow rate of the mobile phase was 0.8 ml/min and the column temperature was set at 30 °C ± 0.1 °C. The peaks were detected at 254 nm. The system was governed by Waters Empower personal computer software.

Identification of the BA was based on their retention times. The calibration curves, i.e. the peak area versus concentration, were linear in the range of concentration between 0.5 and 50 mg/l. The lines of regression calculated have been used to compute the amount of the analytes in samples by interpolation, using external standard method.

To test the validity of the analytical application of the method for the detection of BA in our samples of cheese, some analytical performances were evaluated. Limit of detection, set on the basis of the signal to noise ratio (S/N) of 3, was 5 mg/kg for all the amines, being 8 mg/kg only for 2-phenylethylamine. Precision and accuracy of the method were assessed spiking cheese samples at three different BA concentration levels (10–20–30 mg/kg) for a total of 12 analyses. The within-assay precision (repeatability) came from four consecutive analyses of the spiked samples in the same day and never exceeded 10%. The accuracy, evaluated by recovery experiments and performed on the same spiked samples used for assessing precision, was found to be between 90% and 102% for all the BA. To check if the method was applied efficiently, quality control was carried out on the day of each round analysis by spiking cheese samples with BA and running the analyses, obtaining recoveries always in agreement with the data previously recorded.

2.6. qPCR (quantitative-PCR) for the quantification of tyramine and histamine producing-bacteria

Molecular analyses for the detection of tyrosine decarboxylase (*tdc*) and histidine decarboxylase (*hdc*) genes in randomly

purchased cheeses were carried out as described by Torriani et al. (2008) and Fernández et al. (2006), respectively.

Total DNAs were extracted from cheeses and purified using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc. Carlsbad, CA, USA) according to the manufacturer's instructions with minor modifications, as reported below. Five grams of cheese were homogenized with 45 ml of sodium citrate solution (2% w/v). After centrifugation (10,000 × g, 4 °C, 10 min), pellets were resuspended twice in sodium citrate solution to recover cells in the aqueous phase. Then to obtain a complete cell lysis storage at –20 °C for 20 min was applied. Quantification of total DNA was achieved using a VersaFluor fluorimeter and a Fluorescent DNA Quantitation Kit (Bio-Rad Laboratories, Milan, Italy). The DNA concentration ranged from 0.9 to 32.2 µg/g.

Amplification, detection, and real-time analysis were performed using an iCycler IQ real-time PCR Detection System (Bio-Rad). Each reaction mixture (25 µl) contained 12.5 µl 2XIQ SYBR Green PCR Supermix™ (Bio-Rad), 0.2 µM of each primer (Invitrogen), the template DNA and ultrapure water. Tyramine and histamine-producers were detected using the primers and conditions described by Torriani et al. (2008) and Fernández et al. (2006), respectively. The cycle threshold (C_t) was determined automatically by the instrument and was used for quantitative analyses. Melting temperature analysis of the PCR products was performed to determine the specificity of the PCR reaction. All the samples were processed in triplicate. The detection limits and calibration curves were set for each target gene by using the strains *Enterococcus faecalis* EF37 and *Lactobacillus buchneri* 301 as positive control for the *tdc* and *hdc* genes, respectively, belonging to the Culture Collection of the Department of Food Science (University of Teramo). The above BA producer reference strains were inoculated in skim milk (Oxoid) and, after serial dilution from 10⁸ to 10² cfu/ml, plated on MRS media for viable counting. DNAs from the same 10-fold serial dilutions were extracted and quantified by qPCR to determine C_t values. Standard curves were obtained by plotting the C_t values of the qPCRs performed on the dilution of DNA against the log of cells per milliliters, as determined by plate counts. The qPCR efficiencies were calculated in exponential phase from the given slopes and according to the equation $E = 10^{[1/\text{slope}] - 1}$. In all PCR runs, negative control (sterilized water), positive control and samples were run in triplicate. Sensitivity of qPCR assays was evaluated with reference to other reports (Hierro et al., 2006; Martorell et al., 2005). The C_t values obtained for cell number concentration ranged from 10 to 10⁸ and showed good linearity for triplicate samples of *E. faecalis* EF37 and *L. buchneri* 301 (Tofalo et al., 2012).

The unknown samples were run alongside a set of known standards in order to determine their log cfu/ml. A standard curve was obtained by plotting the C_t values of the known samples against the cfu/ml, from which the unknown cfu/ml was calculated. All the C_t values obtained represent the average of at least three repetitions.

2.7. Statistical analyses

The averages and standard deviations were calculated for each experimental parameter. Descriptive statistics, analyses of variance (ANOVA) pairwise comparisons of mean values was performed using the statistical software STATISTICA for Windows (STAT. version 8.0, StatSoft Inc. Tulsa, OK, USA).

3. Results and discussion

Twelve randomly purchased commercial cheeses produced in Abruzzo region (two from each dairy farm), were analyzed for

microbiota, chemical parameters and BA content by HPLC analysis and qPCR was performed in order to detect tyramine and histamine producing bacteria. Table 1 showed the main technological characteristics of the examined cheeses, such as type of milk, pasteurization, time and temperature of ripening.

3.1. Microbiological composition

Viable counts of aerobic mesophilic bacteria, CNS, enterococci, yeasts, mesophilic and thermophilic lactobacilli and *Enterobacteriaceae* in Abruzzo's Pecorino cheeses, are reported in Table 2. A great variation in the number and distribution of different microbial groups was observed, with a high presence of aerobic mesophilic bacteria, followed by mesophilic and thermophilic lactobacilli. Cheese has a complex microbiota that can be influenced within a variety of factors such as the factory where the cheese is made, the microbial composition and post-contamination of raw milk, and even the period of the year (Bertoni et al., 2001). In general, LAB dominate the adventitious microbiota prevailing in all cheeses. Overall, in the early phase of manufacture non-starter lactic acid bacteria (NSLAB) are present at very low values, whereas during ripening they increase from approximately 2.0 log cfu/g in hygienically produced raw milk cheeses to 6.0 log cfu/g in ripened cheese (Berthier et al., 2001). NSLAB grow at low temperature, are acid-tolerant and tolerate the lack of fermentable carbohydrates, low pH and a_{wv} , and the presence of bacteriocins, which make the environmental conditions very hostile during ripening (Berthier et al., 2001). Coda et al. (2006) reported that many Italian Pecorino cheeses showed NSLAB values higher than 6.0 log cfu/g at the end of ripening, with the only exception concerning Pecorino Umbro and Pecorino di Pienza cheeses, which were manufactured from pasteurized ewes' milk.

Enterococci ranged from 2.0 to 8.0 log cfu/g. In the three cheeses made up with pasteurized milk enterococci ranged from 4.0 to 5.8 log cfu/g. In a previous study on Pecorino di Farindola made with raw milk enterococci ranged from 6.0 to >8.0 log cfu/g (Schirone et al., 2011). Enterococci are one of the most common bacteria in milk constituting the 40.8% of the LAB microbiota (Samelis et al., 2009). The prevalence of enterococci in dairy products is generally related to the unhygienic conditions during the production and processing milk. In any case, Giraffa (2003) reported that their occurrence in food could be unrelated with direct faecal contamination, but they should derive from human or animal faeces or indirectly from contaminated water sources, exterior of animal and/or from milking equipment and bulk storage tank. High levels of enterococci observed in some Abruzzo's cheeses can have an active role during the whole ripening process, and in particular on BA formation (Martuscelli et al., 2005). Enterococci comprise a major part of the fresh cheese curd microbiota and in some cases they are the predominant microorganisms in the fully ripened product. Their levels in different cheeses depend on the extent of milk contamination and their survival and growth under the particular conditions of cheese manufacturing and ripening. The persistence and dominance of enterococci during ripening has been attributed to their wide range of growth temperatures and their high tolerance to heat and salt (Galgano et al., 2001; Sarantinopoulos et al., 2001; Serio et al., 2007).

As regards, *Enterobacteriaceae*, they are associated to the natural microbiota of many dairy products, and together with coliforms are considered indicative of the microbiological quality of cheese. In fact, the contamination of raw milk during milking, unrefrigerated storage and transport, and the possible contamination of cheese during manufacturing seem to be unavoidable (Chaves-López et al., 2006). The numbers of *Enterobacteriaceae* ranged from not detected

Table 2
Viable count (log cfu/g) of the main microbial groups in the cheese samples.

Microbial groups	Samples												
	PA2	PA3	PA4	PA5	PA6	PA7	PA7	RT0	RT1	RT2	RT3	RT4	MR
Aerobic mesophilic bacteria	8.83 ± 0.13 ^d	4.66 ± 0.18 ^e	7.60 ± 0.06 ^b	5.04 ± 0.08 ^a	7.75 ± 0.09 ^b	7.55 ± 0.04 ^b	8.51 ± 0.30 ^c	5.00 ± 0.0 ^a	9.08 ± 0.18 ^d	5.29 ± 0.04 ^a	8.25 ± 0.17 ^c	6.95 ± 0.08 ^f	
CNS*	5.36 ± 0.10 ^e	3.42 ± 0.09 ^b	6.20 ± 0.07 ^f	3.64 ± 0.11 ^c	—	—	—	—	7.04 ± 0.17 ^g	3.34 ± 0.11 ^b	—	4.97 ± 0.11 ^d	
Enterococci	4.81 ± 0.12 ^e	2.17 ± 0.13 ^b	4.00 ± 0.16 ^c	4.36 ± 0.11 ^d	3.97 ± 0.15 ^c	5.81 ± 0.08 ^g	8.00 ± 0.03 ⁱ	—	7.65 ± 0.11 ^h	4.38 ± 0.01 ^d	—	5.59 ± 0.08 ^f	
Yeasts	3.82 ± 0.08 ^e	4.65 ± 0.10 ^{bc}	2.31 ± 0.12 ^d	—	4.69 ± 0.11 ^{bc}	4.62 ± 0.04 ^b	5.81 ± 0.16 ^f	—	—	—	—	4.80 ± 0.10 ^c	
Mesophilic lactobacilli	8.76 ± 0.11 ^f	4.62 ± 0.04 ^f	6.78 ± 0.06 ^{bc}	5.25 ± 0.16 ^g	7.63 ± 0.11 ^h	7.35 ± 0.12 ^e	8.04 ± 0.23 ⁱ	6.95 ± 0.06 ^{cd}	9.21 ± 0.11 ^m	6.39 ± 0.12 ^a	6.62 ± 0.13 ^{ab}	7.12 ± 0.04 ^{de}	
Thermophilic lactobacilli	7.42 ± 0.05 ^b	—	4.97 ± 0.07 ^g	4.18 ± 0.15 ^e	7.15 ± 0.08 ^a	7.00 ± 0.0 ^a	6.79 ± 0.04 ^f	4.47 ± 0.08 ^f	7.39 ± 0.04 ^b	4.00 ± 0.01 ^d	7.04 ± 0.08 ^a	6.00 ± 0.04 ^h	
<i>Enterobacteriaceae</i>	4.74 ± 0.10 ^c	5.44 ± 0.10 ^d	5.98 ± 0.04 ^e	5.77 ± 0.14 ^{bc}	5.57 ± 0.06 ^{bd}	4.32 ± 0.04 ^f	5.70 ± 0.30 ^b	—	—	—	—	4.72 ± 0.06 ^c	

* CNS: Coagulase negative staphylococci.

Values are means ± S.D.; —: absent in 10 g of cheese; data in the same row with different superscript letters are significantly different ($P < 0.05$).

to about 6.0 log cfu/g. *Enterobacteriaceae* are commonly detected in the final product due to their gradual decrease during cheese ripening (Dahl et al., 2000; Hatzikamari et al., 1999; Medina et al., 1991). However, high numbers of *Enterobacteriaceae* have been reported in different Mediterranean cheeses made from ewes' and goats' raw milk after 30 days of ripening (Freitas and Malcata, 2000; Freitas et al., 1995, 1996; Macedo et al., 2004; Nuñez and Martínez-Moreno, 1976; Psoni et al., 2003; Sánchez-Rey et al., 1993). Although large population of this microbial group may endanger the market of ripened cheese (Medina et al., 1991), the specific characteristics, such as taste, aroma and texture, of some artisanal cheeses have been correlated with *Enterobacteriaceae* counts (Dahl et al., 2000; Morales et al., 2004). In Pecorino Abruzzese, a traditional cheese produced in Central Italy, *Enterobacteriaceae* can be detected after 15 days of ripening at levels of 10^3 and 10^5 cfu/g in spring and summer, respectively, and are still present (10^2 cfu/g) after 60 days (Chaves-López et al., 2006). In addition, *Enterobacteriaceae* are generally considered as microorganisms with a high decarboxylase activity, particularly in relation to the production of cadaverine and putrescine (Chaves-López et al., 2006; Suzzi and Gardini, 2003). However, it is difficult to find a straight correlation between microbial counts and BA content in cheese, because amine-producing abilities of different strains of various bacteria differ widely (Chaves-López et al., 2006).

Pathogens such as *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 were absent in all samples.

3.2. Chemical characterization

Table 3 reported some chemical parameters of the different examined Pecorino cheeses. The moisture values ranged from 61.31 to 65.93% (w/w) for Pecorino d'Abruzzo ripened for 3 months, whereas the others ranged from 26.86 to 44.78% (w/w). These last results were similar to that of other Italian Pecorino cheeses (Coda et al., 2006) that showed mean values from 35 to 38.2% (w/w). Four samples of 3 months ripened Pecorino d'Abruzzo showed higher values also for protein and fat in dry matter, pH and ash content. All cheeses were characterized by different pH values from 5.33 to 6.77. Coda et al. (2006) found that pH values of different Pecorino cheeses ranged from pH 4.68 to 5.80.

3.3. Biogenic amines

In all the cheeses made with raw milk BA were present, with a total content ranging from 266.7 to 5860.6 mg/kg, compared to those of cheeses made with pasteurized milk having a total content ranging from 10.3 to 582.4 mg/kg (Table 4). Therefore milk pasteurization could be an important step in the production of cheeses to reduce total BA content. However in the two cheeses made with pasteurized milk, PA4 and PA5, high quantities of tyramine and histamine were detected. On the contrary, in PA2 and PA7 histamine (10.3 mg/kg) and tyramine (10.6 mg/kg) were found in low values, respectively. Many studies related the presence and concentration of BA in cheeses with pasteurization because it reduces the number of decarboxylating microorganisms such as *Enterobacteriaceae* and enterococci (Fernández et al., 2007; Joosten and Northolt, 1989; Novella-Rodríguez et al., 2004; Ordóñez et al., 1997). Other authors attribute the differences in the BA content of pasteurized and non-pasteurized cheeses to the heat sensitivity of certain cofactors, such as pyridoxal 5-phosphate, needed for the aminoacid decarboxylation reaction (Joosten and Northolt, 1989). Recently Ladero et al. (2008) reported that histamine was detected in 53.8% of cheeses made with raw milk compared to 20% of those made with pasteurized milk and also the average histamine concentration was higher in raw milk

Table 3
Chemical characterization of the examined cheeses.

Chemical composition (% w/w)	Samples											
	PA2	PA3	PA4	PA5	PA6	PA7	RT0	RT1	RT2	RT3	RT4	MR
Moisture	35.82 ± 1.99 ^{ad}	33.07 ± 1.33 ^d	26.86 ± 0.83 ^c	36.41 ± 1.64 ^a	37.51 ± 1.57 ^a	28.40 ± 1.07 ^c	44.78 ± 1.16 ^e	61.81 ± 2.39 ^b	62.49 ± 1.63 ^b	65.93 ± 1.44 ^f	61.31 ± 1.35 ^b	42.1 ± 0.75 ^e
Protein in dry matter	38.41 ± 1.97 ^a	35.32 ± 0.61 ^d	39.28 ± 1.64 ^a	40.08 ± 1.16 ^a	40.60 ± 0.41 ^a	38.35 ± 1.30 ^a	38.99 ± 1.57 ^a	63.26 ± 0.68 ^b	79.66 ± 1.55 ^c	80.10 ± 1.41 ^c	62.26 ± 1.18 ^b	39.82 ± 0.53 ^a
pH	5.53 ± 0.04 ^{cd}	5.80 ± 0.0 ^{ef}	6.17 ± 0.16 ^a	6.02 ± 0.0 ^a	5.92 ± 0.07 ^{fg}	5.71 ± 0.06 ^{de}	5.44 ± 0.06 ^{bc}	6.50 ± 0.18 ^h	6.77 ± 0.16 ⁱ	6.21 ± 0.04 ^a	6.56 ± 0.03 ^h	5.33 ± 0.02 ^b
Fat in dry matter	50.19 ± 2.84 ^{eb}	49.86 ± 1.24 ^{ab}	47.65 ± 1.10 ^a	48.81 ± 0.44 ^{ab}	43.03 ± 1.64 ^c	51.79 ± 1.64 ^b	31.08 ± 1.32 ^e	60.85 ± 1.59 ^f	72.65 ± 1.29 ^g	83.71 ± 1.53 ⁱ	76.96 ± 1.39 ^h	45.37 ± 0.49 ^{cd}
α_w	0.930 ± 0.03 ^{ab}	0.950 ± 0.03 ^b	0.920 ± 0.03 ^{abc}	0.890 ± 0.0 ^{ac}	0.870 ± 0.06 ^c	0.910 ± 0.04 ^{abc}	0.930 ± 0.0 ^{ab}	0.930 ± 0.0 ^{ab}	0.910 ± 0.0 ^{abc}	0.90 ± 0.0 ^{abc}	0.930 ± 0.0 ^{ab}	0.910 ± 0.0 ^{abc}
Ash content	7.40 ± 0.42 ^{ab}	6.38 ± 0.40 ^b	8.91 ± 0.49 ^c	7.38 ± 0.52 ^{ab}	7.68 ± 0.08 ^{abc}	7.89 ± 0.0 ^{bc}	8.40 ± 0.53 ^{ac}	13.56 ± 0.49 ^e	17.01 ± 1.58 ^f	17.35 ± 1.15 ^f	12.25 ± 0.64 ^{de}	11.07 ± 0.42 ^d

Data are expressed as mean value ± S.D.; Means within a row with different superscript letters are significantly different ($P < 0.05$).

Table 4
Biogenic amines concentration (mg/kg cheese) in examined cheeses.

Biogenic amines	Samples											
	PA2	PA3	PA4	PA5	PA6	PA7	RT0	RT1	RT2	RT3	RT4	MR
2-Phenylethylamine	nd	232.4 ± 5.66 ^f	nd	26.0 ± 1.70 ^c	62.1 ± 1.70 ^f	nd	12.7 ± 0.99 ^b	nd	44.4 ± 1.98 ^e	nd	nd	nd
Putrescine	nd	986.0 ± 4.24 ^h	8.3 ± 0.28 ^b	8.9 ± 0.28 ^b	36.3 ± 1.27 ^c	nd	128.1 ± 4.38 ^e	nd	377.7 ± 2.40 ^g	369.3 ± 1.70 ^f	55.1 ± 1.41 ^d	127.0 ± 1.99 ^e
Cadaverine	nd	2127.6 ± 2.40 ^g	36.9 ± 1.13 ^c	nd	57.3 ± 0.99 ^d	nd	33.6 ± 1.27 ^b	nd	172.4 ± 3.39 ^f	116.4 ± 1.41 ^e	nd	110.6 ± 2.12 ^e
Histamine	10.3 ± 1.56 ^a	743.3 ± 1.84 ⁱ	200.0 ± 2.26 ^f	235.4 ± 1.27 ^g	13.1 ± 1.70 ^{ab}	nd	130.7 ± 2.97 ^d	266.7 ± 2.40 ^h	186.3 ± 1.84 ^e	14.5 ± 0.99 ^b	761.4 ± 1.13 ⁱ	11.2 ± 0.78 ^a
Tyramine	nd	1771.3 ± 1.84 ⁱ	58.9 ± 0.57 ^c	312.1 ± 2.97 ^f	394.5 ± 0.99 ^g	10.6 ± 0.42 ^b	140.9 ± 4.24 ^d	nd	702.4 ± 2.26 ^h	224.4 ± 1.98 ^e	7.7 ± 0.57 ^b	397.7 ± 0.73 ^g
Total	10.3	5860.6	304.1	582.4	563.2	10.6	446.0	266.7	1483.1	724.7	824.2	678.5

Values are expressed as mean ± S.D; nd: not detected; data within a row with different superscript letters are significantly different ($P < 0.05$). Spermidine and spermine were not detected in all samples (< 5 mg/kg cheese).

cheeses. Only partially these data have been confirmed by the results reported in this paper. It is well known that BA accumulation in cheese can be influenced, firstly by the microbial quality of raw milk, the ripening time, the manufacturing process and the sanitation procedures adopted. An analogous heterogeneity in BA content was observed by Novella-Rodríguez et al. (2003) in cheeses from bovine milk. The microbial population of raw milk can influence BA presence in cheese, even when thermal treatment was applied for Pecorino cheeses (Lanciotti et al., 2007; Martuscelli et al., 2005). In the cheeses PA2, PA4, PA5 and PA7 produced with pasteurized milk, it can be hypothesized that the mild thermal treatment applied selects a decarboxylating microbial population, which dominates during cheesemaking and, possibly, during ripening. Similar results have been reported by Marino et al. (2008) whereas other authors observed a higher BA accumulation in cheeses obtained with raw milk than in similar products made with pasteurized milk (Schneller et al., 1997).

Seven examined cheeses out of twelve had a content of histamine higher than 100 mg/kg. According to the EU Regulation No 2073/2005, only the histamine levels are established for fishery products at 200 mg/kg for fresh fish and up to 400 mg/kg for cured products. While, the US Food and Drug Administration considers histamine a danger to health if its level is equal to 500 mg/kg. Although there are no regulations governing the histamine content in most foodstuffs, some laboratories have made a recommendation to limit the presence of histamine to 100 mg/kg in fermented food products (ten Brink et al., 1990).

3.4. Quantification of histamine and tyramine-producing bacteria in Pecorino cheeses

In the last years, real-time PCR applications to screen and quantify histamine and tyramine-producing bacteria in cheeses have been developed (Ladero et al., 2008, 2010b; Lucas et al., 2008), because quantitative and cost-effective methods are essential to estimate the risks and factors influencing BA accumulation in dairy products. An objective of this study was to determine the histamine and tyramine-producing bacteria in Pecorino cheeses by *hdc* and *tdc* genes qPCR detection, respectively.

Tyramine and histamine producers ranged from 1.57 to 6.35 and from not detected to 7 log cfu/g, respectively (Table 5). Histamine detected by HPLC was present in 11 out of 12 Pecorino cheeses; in particular 5 samples with a quantity ranging from not detected to 14.5 mg/kg and seven with more than 100 mg/kg, ranging from 130.7 to 761.4 mg/kg. The histamine concentration and C_t values were inversely correlated and lower values of C_t were detected in cheeses with higher amounts of histamine (PA3 and RT4). These two cheeses had also the higher presence of histamine producing bacteria, about 10^7 cfu/g. Nevertheless, some cheeses with high histamine producer concentrations but low histamine concentrations were found, suggesting that other factors could influence histamine accumulation. With the exception of RT0 sample having a C_t of 31, it was possible to correlate C_t values ≤ 28 with histamine concentrations > 100 mg/kg, the recommended upper limit for this BA in fermented foods. Similar results have been reported by Ladero et al. (2008) in cheeses from France, Switzerland, Italy, The Netherlands and Spain. In PA6 and RT1 samples histamine producers were absent, this result could be underestimate since efficiency in the recovery of DNA depends on the matrix and on the resistance of the individual strains to the extraction treatments used (Ladero et al., 2010b).

Six Pecorino cheeses showing tyramine concentration of > 200 mg/kg, a recommendable limit, had different C_t values, three with a $C_t \geq 28$ (corresponding to $\geq 10^2$ cfu/g) and three with a $C_t \leq 28$. In fact a C_t value < 28 has been proposed by Ladero et al.

Table 5

BA content and quantification of potential BA-producer microorganisms in examined cheeses.

Samples	Tyramine			Histamine		
	C _t	qPCR (log cfu/g)	HPLC (mg/kg)	C _t	qPCR (log cfu/g)	HPLC (mg/kg)
PA2	34.0	1.57	nd	32.0	2.03	10.3
PA3	15.0	6.35	1771.3	12.4	7.00	743.3
PA4	20.5	4.97	58.9	23.5	4.21	200.0
PA5	32.0	2.03	312.1	28.0	3.08	235.4
PA6	31.1	2.30	394.5	–	–	13.1
PA7	33.0	1.81	10.6	33.0	1.82	nd
RT0	28.3	3.00	140.9	31.0	2.32	130.7
RT1	34.0	1.57	nd	–	–	266.7
RT2	23.0	4.33	702.4	27.3	3.25	186.3
RT3	29.7	2.64	224.4	29.3	2.75	14.5
RT4	18.5	5.47	7.7	14.0	6.60	761.4
MR	23.0	4.33	397.7	32.0	2.03	11.2

nd: not detected; –: <10 cfu/g.

(2010b) as threshold limit for qPCR. In some cases a linear correlation between high tyramine-producing microorganism and high tyramine concentrations wasn't found. Our results together with those obtained by Ladero et al. (2010b) demonstrated that 10^3 – 10^4 cfu of tyramine producing bacteria per g of cheese are the minimum concentration of cells to detect significant amount of this BA. Tyramine content depends on the requirement of a minimum of tyramine-producing microorganisms, as well as on appropriate environmental conditions, such as tyrosine availability.

Even if there are some unexpected results, qPCR could be very useful to identify early cheeses contaminated with histamine and tyramine producers that could accumulate these amines during the ripening period yielding unsafe products. The presence and quantification of tyramine and histamine producers could be detected prior to the accumulation, even during cheese manufacture (Ladero et al., 2008). This could be particularly important for Pecorino cheeses in which it is often possible to detect high levels of these two BA (Schirone et al., 2011).

4. Conclusions

Although the studied Pecorino cheeses were all from the same area (Abruzzo region), differences were observed for the microbiological profiles and BA contents. Many factors can affect the growth and the biochemical activities of cheese microbiota involved in BA production such as different degrees of hygienic quality of raw milk and the different handling and cheesemaking processes. The wide distribution of *tdc* positive bacteria detected in all the cheeses and *hdc* positive ones found in ten cheeses, suggest that the two BA could reach toxicologically relevant levels in all analyzed Pecorino cheeses in few months of ripening. Information regarding BA content in traditional cheeses is very important for the food trade sector because recommended upper levels of BA content vary among countries. At the moment the consumption of these cheeses by patients using MAO inhibitors should be reduced or avoided.

Even if HPLC analysis is essential to determine the exact content of tyramine and histamine in the cheese, qPCR is an easy and rapid method for analyzing many samples and can reduce the number of the samples for analysis by HPLC and detect the presence of BA producers even when BA are still undetectable; this early detection could allow an appropriate action to avoid negative impacts. In fact, in Spain this approach was patented (ES2270650) to detect tyramine LAB producers. Because of advantages offered by qPCR it

could be applied to traditional cheeses to improve their safety and quality.

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