A survey of the main technology, biochemical and microbiological features influencing the concentration of biogenic amines of twenty Apulian and Sicilian (Southern Italy) cheeses

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ABSTRACT

Twenty Apulian and Sicilian cheeses were analysed for their concentrations of eight biogenic amines (BAs), free amino acids, pH, water activity, and subjected to microbiological characterisation. In addition, lactic acid bacteria isolated from cheeses were assayed for their capacity to generate BAs. Principal component analysis was performed to find the effect of different parameters on the distribution of the cheeses. Although short-ripened ($\leq$30 d) cheeses did not show significant BA concentrations, the only BA showing high positive correlation with time of ripening was histamine. Concentration of histidine and, especially, percentage of histidine-decarboxylase bacteria presumably affected histamine concentration. High pH values were negatively correlated to the concentration of tyramine, putrescine, and cadaverine. Fifty percent of the cheeses contained at least one BA at potentially toxic concentrations. Unambiguous and ever-valid relations among parameters and BAs are difficult to determine, because BAs are the result of combined and varied factors.

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1. Introduction

Biogenic amines (BAs) are low-molecular nitrogenous organic bases that are formed in foodstuffs by microbial decarboxylation of the precursor amino acids. Tyrosine, histidine, lysine, tryptophan, and phenylalanine are, respectively, precursor amino acids for tyramine, histamine, cadaverine, tryptamine, and 2-phenylethylamine. Ornithine and arginine may be the precursors for putrescine, spermidine and spermine. Although small amounts of BAs are biosynthesised in plant and animal cells, having different biological activities (Pinho et al., 2004), these compounds are potentially toxic to human health. The effects on nervous and vascular systems are particularly severe in sensitive people or when the amine oxidases, naturally involved in the detoxification, are inhibited (Shalaby, 1996; Silla Santos, 1996). The toxicity limit of BAs in foods is estimated to be 100 mg kg^{-1}, even though it is stated that the safe sum of histamine, tyramine, putrescine and cadaverine should not exceed 900 mg kg^{-1} (Shalaby, 1996; Valsamaki, Michaelidou, & Polychroniadou, 2000).

Cheeses are among the foods most commonly associated with the presence of BAs (Innocente & D'Agostin, 2002; Moret, Bortolomeazzi, Feruglio, & Lerker, 1992; Stratton, Hutkins, & Taylor, 1991). Indeed, the main biochemical process that takes place during cheese ripening, proteolysis, leads to the accumulation of free amino acids (FAAs), some of which are precursors of BAs. The BA concentration of many typical and/or traditional Italian cheeses was analysed (Innocente, Biasutti, Padovese, & Moret, 2007; Ladero, Fernández, & Álvarez, 2009; Martuscelli et al., 2005; Schirone et al., 2013; Spizzirri et al., 2013). Overall, the concentration and type of BAs in cheeses is extremely variable, depending on: (i) type of milk (cows'/sheep's/goats' milk); (ii) thermal treatment of cheese milk; (iii) section of the cheese (edge/core); (iv) ripening conditions; (v) post-ripening processing; (vi) type of packaging; (vii) storage time and temperature; and (viii) microbiota responsible for cheese-making (Loizzo et al., 2013). Generally, the concentration of BAs was lower in short-ripened than in long-ripened cheeses (Bunková et al., 2010; Fernández, Linares, Del Rio, Ladero, & Alvarez, 2007), where the level of proteolysis and catabolism of

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FAAs increases. Cheeses possess very rich, diverse and complex microbiota, mainly deriving from primary starter lactic acid bacteria and adventitious non-starter lactic acid bacteria (NSLAB; Beresford & Williams, 2004). High cell densities of NSLAB, possessing amino acid decarboxylating enzymes (e.g., tyrosine decarboxylase), were positively correlated with high content of BAs in cheeses (Bunková et al., 2010; Fernández et al., 2007; Komprda et al., 2008; Ladero et al., 2009; Martuscelli et al., 2005). Nevertheless, the concentration of ethylamine, tryptamine, 2-phenylethylamine, and cystamine during ripening of Terrincho cheese, reached the maximum at 30 days, but subsequently decreased, meaning that such compounds are also degraded or transformed (Pinho et al., 2004).

Although, except for histamine in fish products, there is no consensus on the maximum permitted concentration of BAs in foods, everybody acknowledges that reducing the concentration of such potentially toxic compounds in foods is extremely important. The formation of BAs may be limited through the use of either amine-negative (not able to decarboxylate FAAs into BAs) or amine oxidising starter cultures (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2000, 2001; Latorre-Moratalla et al., 2007; Mah, Kim, & Hwang, 2009; Spicka, Kalac, Bover-Cid, & Krizek, 2002; Stratton et al., 1991).

To the best of our knowledge, no studies were focused on the concentration of all of the possible BAs in a high number of traditional cheeses, and looking at this food safety feature as possibly affected by technology parameters, concentration of precursor FAAs and microbiological characteristics. Therefore, the aim of this study was to correlate the content of BAs of twenty traditional Apulian or Sicilian (Southern Italy) cheeses with several technological and microbiological features such as time of ripening, pH, concentration of precursor FAAs, and occurrence of decarboxylase-positive lactic acid bacteria.

2. Materials and methods

2.1. Cheeses

Nine Apulian (Cacio, Caciocavallo Podolico Dauno, Caciocavallo Silano Protected Designation of Origin (PDO), Cacioricotta, Canestrato Pugliese PDO, Caprino di Biccari, Caprino di Castel Fiorentino, Pecorino Foggiano, and Vaccino), and eleven Sicilian (Caciocavallo Palermitano, Ragusano PDO, Caprino Girgentano, Fior di Capra, Fiore Sicano, Maiorchino, Pecorino Siciliano PDO, Piacentinu Ennese, Provola dei Nebrodi, Tuma Persa, and Vastedda della valle del Belice PDO) traditional cheeses were evaluated in this study. Table 1 summarises the main parameters used during manufacturing and ripening, as well as the approximate moisture level of each cheese. Three batches of each cheese were collected at local dairy farms and kept at 4 °C during transfer (1—3 h) to laboratory. Each cheese was analysed for BAs, pH, water activity (a_w), FAAs, and microbiological features.

2.2. Determination of biogenic amines in cheeses

BAs were extracted from cheese, derivatised and quantified, according to the method described by Innocente et al. (2007), with modifications. In detail, 10 g of cheese were weighed in a 50 mL polypropylene tube and 20 mL of 0.1 mol L^{-1} HCl added, containing 1,7-diaminoheptane (0.01 mg mL^{-1}) as the internal standard. The suspension was homogenised for 2 min in a BagMixer 400P (Interscience, St. Nom, France) blender. The homogenate was centrifuged at 15,557 × g, at 4 °C, for 30 min and the supernatant (first acid extract) was transferred into a clean 50 mL polypropylene tube. The pellet was added with 20 mL of 0.1 mol L^{-1} HCl, containing the internal standard, homogenised and centrifuged as described above. The supernatant (second acid extract) was

recovered and mixed with the first acid extract. The two extracts were diluted to 50 mL with 0.1 mol L^{-1} HCl.

Extracted BAs were derivatised by mixing 1 mL of extract, 0.5 mL of saturated $NaHCO_3$ solution and 1 mL of dansyl chloride (DCl) reagent (dissolved in acetone at 10 g L^{-1}) in a 15 mL polypropylene tube, protected from light. The reaction mixture was then left for 60 min at 40 °C and vortexed at 15 min intervals. Excess of DCl was removed by addition of 0.3 mL of ammonia solution (300 g L^{-1}), vortexing for 1 min and leaving to react in the dark for 15 min at room temperature. The sample was extracted twice (duration of each extraction: 5 min) with 1 mL aliquot of diethyl ether. The combined extracts were collected in 2 mL polypropylene tubes and dried (110 min, room temperature) in a vacuum centrifuge (SpeedVac Concentrator SPD121P, Thermo Fisher Scientific, Marietta, OH, USA). Finally, the residue was re-dissolved in 1 mL of acetonitrile for injection.

BAs were separated using an Äkta Purifier 10 (GE Healthcare Bio-Sciences, Uppsala, Sweden), equipped with a 20 µL loop, a reverse phase C_{18} column (Kromasil 100 A, 5 µm, 4.6 × 250 mm, StepBio, Bologna, Italy) thermostated at 30 °C, with a guard cartridge Kromasil 100-5C18, and a UV detector at 254 nm. BAs were eluted at 0.8 mL min^{-1} with acetonitrile (A) and water (B), using the following gradient: 65% A (1 min), 65—80% A (9 min), 80—90% A (2 min), 90—100% A (4 min), 100% A (7 min) (Moret, Smela, Populin, & Conte, 2005). BAs were quantified using calibration curves built up after having analysed standard solutions, containing the following amines dissolved in 0.1 M HCl at concentrations of 2, 5, 10, and 20 µg mL^{-1}: cadaverine, histamine, 2-phenylethylamine, putrescine, spermidine, spermine, tryptamine and tyramine. The following formula was used:

$$\text{Concentration of BAs} \left(\text{mg kg}^{-1}\text{of cheese}\right)$$
$$= [(\text{BA peak area}/\text{internal standard peak area}) - q]/m$$

where q and m are the parameters of the calibration curve for that amine. Prior to HPLC analysis, the standard solutions were derivatised under the same conditions as the acid extracts, except for the concentration of DCl (5 g L^{-1}).

2.3. Determination of pH, water activity, and free amino acids in cheeses

The value of pH was determined by direct insertion of a Foodtrode (Hamilton, Bonaduz, Switzerland) electrode. Water activity (a_w) was determined using the Dew Point Water Activity Meter AquaLab (Mod. 4TE, Decagon Devices, Inc., Pullman, WA, USA), according to the manufacturer's instructions.

Concentration of individual FAAs in cheese was determined as described by Siragusa et al. (2007), with few modifications. In detail, 30 g of cheese was grated and homogenised (5 min treatment) with 90 mL of 50 mmol L^{-1} phosphate buffer, pH 7.0, in a blender. The suspension was kept at 40 °C for 1 h under gentle stirring (150 rpm) and centrifuged at 1157 × g for 30 min at 4 °C. The supernatant was collected and centrifuged (1157 × g, 10 min, 4 °C). One millilitre of the supernatant from the second centrifugation was added with 50 mg of cold sulphosalycillic acid (final concentration: 50 mg mL^{-1}) and incubated for 1 h at 4 °C to precipitate proteins and most of peptides. After centrifugation (23,000 × g, 15 min), the extract, containing just FAAs, was filtered (Mini-Uni PrepTM, pore size 0.2 µm, GE Healthcare Life Science), diluted (if needed) with sodium citrate loading buffer, and injected (20 µL) into a Biochrom 30 amino acid analyser (Biochrom Ltd., Cambridge, UK), equipped with a sodium cation-exchange column (20 by 0.46 cm [inner diameter]). Amino acids were post-column

Table 1
Parameters used during manufacture and ripening and approximate moisture levels of traditional Apulian or Sicilian cheeses.

Cheese	Code	Location of the dairy factory[a]	Type of milk[b]	Milk thermal treatment	Type of rennet	Type of starter culture[c]	Curd thermal treatment (temperature, time)	Type of salting	Ripening time (days)	Ripening conditions[d]	Moisture (%, w/w)
Vastedda della Valle del Belice PDO	C1	Contessa Entellina (PA), S	E	None	Lamb, paste	None	Stretching (80 °C, 5 min)	Brine	2	nc	55.0
Caprino di Castel Fiorentino	C2	Torremaggiore (FG), A	G	None	Calf, liquid	Natural milk starter culture	None	Dry	30	10–15, nc	43.5
Caciocavallo Podolico Dauno	C3	Torremaggiore (FG), A	C	None	Calf, liquid	Natural whey starter culture	Stretching (80 °C, 5 min)	Brine	30	10–15, nc	41.9
Fior di Capra	C4	Campobello di Licata (AG), S	G	None	Lamb/kid/plant paste	None	None	Dry	90	nc	41.9
Caprino Girgentano	C5	Campobello di Licata (AG), S	G	None	Plant (artichoke)	None	None	Dry	90	nc	40.0
Caprino di Biccari	C6	Biccari (FG), A	G	Thermisation	Lamb and calf, liquid	Commercial	None	Dry	210	11–12, 70	38.4
Caciocavallo Palermitano	C7	Godrano (PA), S	C	None	Lamb, paste	None	Stretching (80 °C, 5 min)	Brine	120	14–16, nc	37.0
Ragusano PDO	C8	Ragusa (RG), S	C	None	Lamb, paste	None	Stretching (80 °C, 5 min)	Brine	120	14–16, nc	36.9
Caciocavallo Silano PDO	C9	Putignano (BA), A	C	None	Calf, paste	Natural whey starter culture	Stretching (85–90 °C, 5 min)	Brine	150	12–15, 75–80	36.5
Cacioricotta	C10	Putignano (BA), A	C	Pasteurisation	Calf, liquid	None	None	Dry	30	20, nc	36.4
Fiore Sicano	C11	Castronovo di Sicilia (PA), S	C	None	Kid, paste	None	Stewing (40 °C)	Dry	60	9–13, 80–90	36.2
Cacio	C12	Casarano (LE), A	C & E	Pasteurisation	Kid, liquid	Commercial	None	Dry	60	12–14, 40	36.0
Vaccino	C13	Casarano (LE), A	C	Pasteurisation	Kid, paste	None	None	Brine	90	12–14, 40	35.0
Provola dei Nebrodi	C14	Mistretta (ME), S	C	Thermisation	Lamb/kid, paste	None	Stretching (80 °C, 5 min)	Brine	30	nc	34.5
Pecorino Foggiano	C15	Biccari (FG), A	E	Thermisation	Lamb and calf, liquid	Commercial	None	Dry	120	11–12, 70	34.2
Canestrato Pugliese PDO	C16	Biccari (FG), A	E	None	Calf, liquid	None	None	Dry	90	11–12, 70	34.0
Maiorchino	C17	Mistretta (ME), S	E & G	Thermisation	Lamb/kid, paste	None	Cooking (80 °C, 1 h)	Dry	180	nc	33.3
Pecorino Siciliano PDO	C18	Salemi (TP), S	E	None	Lamb, paste	None	None	Dry	120	nc	31.5
Piacentinu Ennese	C19	Enna (EN), S	E	None	Lamb, paste	None	None	Dry	270	nc	29.8
Tuma Persa	C20	Castronovo di Sicilia (PA), S	C	Thermisation	Kid, paste	None	None	Dry	270	nc	27.5

[a] The letter after brackets indicates the region of origin of the cheese: A, Apulia; S, Sicily.
[b] Types of milk were: E, ewe; G, goat; C, cow.
[c] Where used, starters were thermophilic, except for those used for Caprino di Biccari and Pecorino Foggiano, which contained both thermophilic (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Streptococcus thermophilus*) and mesophilic (*Lactobacillus casei*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*) cultures.
[d] First number indicates the ripening temperature (°C), the second number indicates the relative humidity (%) of the ripening cellar; when either ripening temperature or relative humidity were not controlled (nc), cheeses were kept in cool and/or well-ventilated place.

derivatised with ninhydrin reagent and detected by absorbance at 440 (proline) or 570 (all the other amino acids) nm. FAAs were quantified in the extracts directly through EZChrom Elite version 2.8.3 (Scientific Software Inc., Pleasanton, CA, USA), using amino acid standard solutions containing all the amino acids, each one at concentrations of 31.25, 62.50, 125, and 250 mmol L^{-1}.

2.4. Microbiological analyses and isolation of lactic acid bacteria from cheeses

Cheeses were processed as follows: 10 g of cheese were homogenised (3 min treatment) with 90 mL of sodium citrate (20 g L^{-1}) solution in a blender. Serial decimal dilutions of homogenates were made in peptone water (NaCl 8.5 g L^{-1}, peptone 10 g L^{-1}) and plated onto the following media (Oxoid, Basingstoke, UK) for viable counts: Plate Count (PC) agar for total mesophilic aerobic bacteria at 30 °C for 48 h; De Man, Rogosa and Sharpe (MRS) agar for presumptive mesophilic and thermophilic lactobacilli at 25 °C and 42 °C for 48 h under anaerobiosis, respectively; M17 agar for mesophilic lactococci and thermophilic streptococci at 25 °C and 42 °C for 48 h under anaerobiosis, respectively; Violet Red Bile Glucose (VRBG) agar for *Enterobacteriaceae* at 37 °C for 24 h.

For each medium and condition used for enumerating presumptive lactic acid bacteria, a number of colonies equal to the square root of the total number recorded in plates coming from the highest dilution were randomly selected. Gram-positive, catalase-negative, non-motile rods and cocci isolates were cultivated onto MRS or M17 broth at 25 °C (mesophilic) or 42 °C (thermophilic) for 24 h and re-streaked onto the same agar media. All isolates considered for further analyses were able to acidify the culture medium. Stock cultures were stored at −80 °C in 10% (v/v) glycerol.

2.5. Decarboxylase activities of lactic acid bacteria isolated from cheeses

Lactic acid bacteria isolated from cheeses were screened for their capacity to generate BAs through decarboxylation of precursor amino acids, mainly according to the method described by Bover-Cid and Holzapfel (1999). Isolates were pre-cultured twice in MRS or M17 broth containing the precursor amino acid (1 g L^{-1}) and pyridoxal-5-phosphate (0.05 g L^{-1}) as an essential cofactor. Decarboxylase medium (pH 5.3) had the following composition (g L^{-1}): tryptone (5), yeast extract (5), LAB-Lemco (5), sodium chloride (2.5), glucose (0.5), Tween 80 (1), magnesium sulphate (0.2), manganese sulphate (0.05), iron sulphate (0.04), calcium carbonate (0.1), ammonium citrate (2), thiamine (0.01), precursor amino acid (10), dipotassium phosphate (2), pyridoxal-5-phosphate (0.05), bromocresol purple (0.06). Two hundred microlitres of decarboxylase medium were inoculated (4%, v/v) with pre-culture into the well of 96-well plate (CytoOne, Starlab s.r.l., Milan, Italy). Decarboxylase medium without precursor amino acid was also inoculated with each isolate (negative control). After incubation at 37 °C for 96 h, isolates showing colour transition to violet and no colour transition in the negative control were recorded as decarboxylase-positive. Decarboxylase activity was also assessed by inoculating positive isolates (five for each enzyme activity) into MRS or M17 broth supplemented with precursor amino acid (2.5 g L^{-1}; Joosten & Northolt, 1989). Following incubation (25 °C or 42 °C, 48 h), BA concentration was determined as described in Section 2.2.

2.6. Statistical analyses

Data (at least three biological replicates) of pH, a_w, concentrations of BAs and FAAs, and cell density of different microbial groups were subjected to pair−comparison of treatment means by Tukey's procedure at $P < 0.05$, using the statistical software Statistica 7.0 for Windows (StatSoft Italia srl, Vigonza, Italy). Principal Component Analysis (PCA), using a correlation matrix, was carried out to find the effect of different parameters on the distribution of the cheeses.

3. Results

3.1. Concentration of biogenic amines

Results concerning biogenic amine contents are presented in Table 2. The sum of cadaverine, histamine, putrescine and tyramine in cheeses was highly variable, with a median value of 137.1 mg kg^{-1}. Pecorino Foggiano (C15) was the only cheese exceeding the concentration of 900 mg kg^{-1}. However, Caciocavallo Silano PDO (C9), Canestrato Pugliese PDO (C16), Caprino di Biccari (C6) and Piacentinu Ennese (C19) cheeses contained high (>500 mg kg^{-1}) total concentrations of cadaverine, histamine, putrescine and tyramine. Conversely, nine cheeses, including Caciocavallo Podolico Dauno (C3), Provola dei Nebrodi (C14) and Vastedda della valle del Belice PDO (C1), showed a low (<100 mg kg^{-1}) total concentration.

Overall, the number of Apulian cheeses containing high concentrations of tyramine and putrescine was higher than Sicilian cheeses, whereas the opposite was found for histamine. Tyramine was the only BA detected in all the cheeses, although at extremely variable concentrations (Table 2). The median value for tyramine was 27.15 mg kg^{-1}. Cacio (C12), Canestrato Pugliese PDO (C16) and Pecorino Foggiano (C15) were characterised by high concentrations (273−305 mg kg^{-1}) of tyramine. Histamine median value was 31.5 mg kg^{-1}. It was not detected in Caciocavallo Podolico Dauno (C3) and Caprino di Castel Fiorentino (C2) cheeses. The median value for putrescine was 15.7 mg kg^{-1}. Pecorino Foggiano (C15), Caprino di Biccari (C6), Canestrato Pugliese PDO (C16) and Piacentinu Ennese (C19) cheeses contained largely more than 100 mg of putrescine per kg. Caciocavallo Podolico Dauno (C3) was the only cheese not containing a detectable concentration of putrescine. The median value for cadaverine was 4.15 mg kg^{-1}. Canestrato Pugliese PDO (C16), Pecorino Foggiano (C15) and Cacio (C12) cheeses contained cadaverine at concentrations higher than 100 mg kg^{-1}. Conversely, cadaverine was not detected in Caciocavallo Palermitano (C7), Caprino di Castel Fiorentino (C2) and Tuma Persa (C20) cheeses. 2-phenylethylamine varied from 0 to ca. 145 mg kg^{-1} (Table 2), with a median value of 3.3 mg kg^{-1}. Caprino di Biccari (C6), Cacio (C12) and Maiorchino (C17) showed the highest concentration of 2-phenylethylamine.

Considering 100 mg kg^{-1} as the limit of concentration of each individual BA in cheeses, histamine, tyramine, and putrescine exceeded this limit in the highest number of cheeses (seven, five, and four, respectively), more than for cadaverine and 2-phenylethylamine. Tryptamine and, especially, spermidine and spermine were either not detected or present at concentration lower than 100 mg kg^{-1}. Five cheeses (Cacio, Canestrato Pugliese PDO, Caprino di Biccari, Pecorino Foggiano, and Piacentinu Ennese) showed concentration higher than 100 mg kg^{-1} not only for tyramine but also for other two or three BAs. Five cheeses (Caciocavallo Palermitano, Caciocavallo Silano PDO, Fiore Sicano, Pecorino Siciliano PDO, and Tuma Persa) were characterised by high concentration of only histamine. The remaining cheeses, including Apulian and Sicilian types, contained BAs at individual concentration lower than 100 mg kg^{-1} (Table 2).

3.2. pH, water activity and concentration of free amino acids

The values of pH and a_w varied from approximately 4.93 (Canestrato Pugliese PDO, C16) to ca. 5.71 (Provola dei Nebrodi,

Table 2
Concentration of biogenic amines in traditional Apulian or Sicilian cheeses.[a]

Cheese[a]	Cadaverine	Histamine	2-Phenylethylamine	Putrescine	Spermidine	Spermine	Tryptamine	Tyramine	Total[b]
C1	1 (0.1)	5.7 (0.4)	0.5 (0.1)	1.1 (0.1)	0.3 (0.2)	1.1 (0.9)	1.4 (0.2)	5.9 (0.1)	13.7
C2	nd	nd	nd	9 (3)	nd	4.7 (0.3)	nd	25 (3)	34
C3	1.4 (0.2)	nd	nd	nd	nd	13.6 (0.4)	nd	7 (1)	8.4
C4	0.3 (0.0)	18 (5)	nd	15.4 (0.6)	nd	nd	0.8 (0.2)	29.3 (0.2)	63
C5	0.9 (0.0)	89 (9)	4 (0.0)	6.2 (0.7)	nd	nd	0.4 (0.1)	1.7 (0.1)	97.8
C6	27 (2)	44 (3)	145 (18)	298 (17)	5 (3)	nd	73 (12)	227 (26)	596
C7	nd	149 (3)	nd	12 (4)	13 (5)	nd	4 (1)	4 (3)	165
C8	2.9 (0.0)	54.8 (0.3)	4.5 (0.0)	7.6 (0.3)	nd	nd	nd	23.9 (0.1)	89.2
C9	17 (0.7)	435 (35)	nd	37 (5)	nd	nd	6 (1)	33 (4)	522
C10	18 (5)	19 (5)	nd	14 (4)	5 (2)	10 (0.5)	nd	13 (4)	64
C11	32 (1)	155 (22)	2.6 (0.5)	16 (3)	nd	nd	53 (16)	32 (5)	235
C12	111 (23)	17 (3)	143 (16)	53 (9)	nd[b]	nd	18 (2)	305 (55)	486
C13	28 (1)	14.9 (0.3)	nd	62 (6)	nd	15(3)	nd	4.3 (0.6)	109.2
C14	0.4 (0.0)	1 (0.0)	0.1 (0.0)	0.75 (0.03)	0.10 (0.00)	nd	0.1 (0.0)	1.0 (0.1)	3.2
C15	129 (27)	253 (30)	25 (11)	594 (11)	nd	nd	nd	303 (26)	1279
C16	199 (19)	3.9 (0.4)	46 (3)	208 (19)	4 (2)	nd	nd	273 (28)	683.9
C17	1.3 (0.1)	2.0 (0.7)	82 (5)	5 (0.0)	3.5 (4.1)	nd	1.9 (0.2)	61 (18)	69.3
C18	5.4 (0.3)	219 (11)	9 (1)	60 (3)	2 (0.0)	nd	2.8 (0.4)	86 (17)	370.4
C19	9.5 (1)	243 (6)	28.5 (1)	204 (1)	10 (2)	nd	6 (1)	134 (9)	590.5
C20	nd	250 (16)	4 (0.5)	23 (3)	nd	nd	2.3 (1.4)	14 (0.1)	287

[a] Values (in mg kg^{-1}) are means with standard deviation in parentheses; nd, not detected. For cheese codes C1–C20, see Table 1.
[b] Sum of cadaverine, histamine, putrescine, and tyramine.

C14), and from approximately 0.73 (Cacioricotta, C10) to approximately 0.99 (Vastedda della valle del Belice PDO, C1), respectively (Table 3). The median values for pH and a_w were 5.40 and 0.93, respectively. Most of the cheeses had an intermediate acidity (4.5 < pH < 5.5), with the exceptions of Cacio (C12), Ragusano PDO (C8), Pecorino Siciliano PDO (C18), Piacentinu Ennese (C19), Provola dei Nebrodi (C14), and Vastedda della valle del Belice PDO (C1), which showed higher values of pH. Except for Cacioricotta (C10), Canestrato Pugliese PDO (C16), Pecorino Foggiano (C15) and Piacentinu Ennese (C19) cheeses, which showed values of a_w lower than 0.90, all the other cheeses had an intermediate to high value of a_w.

The concentration of total FAAs ranged from 622 (Cacioricotta) to 12,779 (Tuma Persa) mg kg^{-1}, with a median value of 4524.5 mg kg^{-1}. The total concentration of FAA precursors of BAs varied in the interval 216–4275 mg kg^{-1}, the median value being 1479.5 mg kg^{-1}. Tuma Persa was by far the cheese characterised by the highest concentration of total FAAs potentially precursors of BAs,

followed by Caciocavallo Silano PDO (C9), Caprino di Biccari (C6) and Piacentinu Ennese (C19) (approximately 2500–2900 mg kg^{-1}) (Table 3). Ragusano PDO, Cacioricotta and Provola dei Nebrodi cheeses showed the lowest concentration of total precursor FAAs (approximately 200–300 mg kg^{-1}), whereas the other cheeses had intermediate values. Among individual FAAs, lysine and arginine were present at the highest (452 mg kg^{-1}) and the lowest (57 mg kg^{-1}) median values, respectively. Tyrosine, phenylalanine, and ornithine were found at highest concentration in Tuma Persa cheese. Histidine and arginine showed the highest concentrations in Caciocavallo Silano PDO (Table 3).

3.3. Microbiological analyses and free amino acid-decarboxylase activities of presumptive lactic acid bacteria

When detected, *Enterobacteriaceae* was the microbial group found at the lowest cell density. *Enterobacteriaceae* ranged from approximately 2.0 log cfu g^{-1} (Fiore Sicano, C11) to approximately

Table 3
pH, water activity and concentration (mg kg^{-1}) of total and precursor free amino acids of traditional Apulian or Sicilian cheeses.[a]

Cheese	pH	Water activity	Free amino acids								
			Total	Tyrosine	Phenylalanine	Histidine	Tryptophan	Ornithine	Lysine	Arginine	Precursor
C1	5.57 (0.02)	0.99 (0.00)	1161	31 (2)	110 (7)	15 (2)	19 (2)	125 (7)	109 (7)	8 (2)	417
C2	5.31 (0.02)	0.90 (0.01)	3230	142 (7)	264 (12)	68 (5)	168 (7)	36 (3)	275 (13)	82 (5)	1035
C3	4.95 (0.03)	0.90 (0.02)	2871	109 (7)	210 (10)	94 (7)	117 (10)	10 (4)	338 (18)	262 (15)	1140
C4	5.30 (0.01)	0.93 (0.00)	3901	164 (14)	507 (45)	69 (7)	158 (17)	392 (40)	429 (40)	31 (4)	1750
C5	5.29 (0.01)	0.94 (0.01)	5618	282 (20)	505 (31)	108 (7)	164 (11)	182 (10)	521 (32)	251 (16)	2013
C6	4.99 (0.01)	0.94 (0.01)	9074	107 (8)	750 (53)	222 (15)	301 (24)	24 (3)	1170 (88)	173 (15)	2747
C7	5.44 (0.00)	0.97 (0.00)	2076	76 (8)	139 (11)	45 (6)	59 (5)	22 (3)	201 (17)	64 (6)	606
C8	5.51 (0.01)	0.97 (0.01)	779	23 (5)	88 (10)	19 (2)	25 (4)	39 (2)	83 (5)	nd	277
C9	5.04 (0.00)	0.93 (0.01)	8708	241 (16)	441 (30)	380 (26)	75 (6)	10 (4)	1016 (75)	428 (31)	2591
C10	5.25 (0.02)	0.73 (0.01)	622	15 (2)	57 (18)	8 (3)	44 (5)	6 (1)	64 (4)	22 (2)	216
C11	5.46 (0.00)	0.96 (0.01)	1259	26 (2)	175 (8)	68 (4)	36 (2)	106 (6)	92 (4)	18 (2)	521
C12	5.58 (0.01)	0.95 (0.01)	4442	68 (3)	342 (14)	112 (8)	194 (10)	171 (7)	476 (19)	65 (3)	1428
C13	5.37 (0.03)	0.97 (0.02)	4607	78 (8)	358 (33)	128 (13)	240 (21)	177 (18)	483 (44)	67 (8)	1531
C14	5.71 (0.01)	0.97 (0.00)	762	46 (2)	64 (3)	14 (2)	63 (4)	47 (2)	59 (3)	20 (1)	313
C15	5.14 (0.01)	0.89 (0.01)	4629	67 (5)	398 (30)	64 (5)	163 (12)	17 (2)	549 (42)	49 (4)	1307
C16	4.93 (0.02)	0.84 (0.02)	5126	106 (11)	432 (36)	99 (12)	271 (26)	126 (1)	415 (35)	131 (13)	1580
C17	5.46 (0.00)	0.93 (0.01)	78,88	46 (4)	586 (46)	137 (11)	203 (18)	545 (48)	817 (75)	nd	2334
C18	5.56 (0.00)	0.91 (0.00)	8332	153 (7)	419 (19)	89 (5)	117 (6)	254 (11)	1086 (55)	nd	2118
C19	5.51 (0.01)	0.89 (0.02)	11,569	167 (10)	830 (49)	127 (6)	182 (11)	106 (6)	1397 (80)	139 (8)	2948
C20	5.50 (0.00)	0.90 (0.01)	12,779	377 (35)	1108 (91)	264 (25)	272 (26)	682 (66)	1522 (147)	50 (5)	4275

[a] For cheese codes C1–C20, see Table 1. Values are means with standard deviation in parentheses; nd, not detected. Precursor free amino acids are those responsible for the synthesis of biogenic amines.

Table 4
Cell densities of total mesophilic aerobic bacteria, presumptive mesophilic and thermophilic lactobacilli, presumptive mesophilic and thermophilic cocci (lactococci/streptococci), and *Enterobacteriaceae* in traditional Apulian or Sicilian cheeses.

Cheese[a]	Total mesophilic aerobic bacteria	Mesophilic lactobacilli	Thermophilic lactobacilli	Mesophilic lactococci	Thermophilic streptococci	*Enterobacteriaceae*
C1	8.3 (0.4)	7.5 (0.5)	7.8 (0.4)	8.5 (0.5)	8.7 (0.4)	2.8 (0.2)
C2	4.9 (0.3)	8.7 (0.1)	7.6 (0.0)	8.9 (0.1)	8.7 (0.2)	4.7 (0.3)
C3	4.2 (0.2)	6.4 (0.1)	5.8 (0.8)	7.3 (0.0)	8.6 (0.1)	<1.0
C4	7.9 (0.4)	7.6 (0.0)	4.9 (0.1)	7.5 (0.1)	5.0 (0.1)	<1.0
C5	6.4 (0.1)	6.9 (0.1)	6.1 (0.0)	6.8 (0.2)	6.8 (0.3)	<1.0
C6	7.2 (0.1)	7.2 (0.1)	7.2 (0.2)	7.0 (0.3)	6.6 (0.1)	<1.0
C7	6.7 (0.3)	6.6 (0.4)	5.8 (0.2)	6.4 (0.4)	5.6 (0.3)	2.5 (0.2)
C8	8.8 (0.2)	7.0 (0.1)	7.2 (0.3)	7.2 (0.3)	7.5 (0.2)	<1.0
C9	6.4 (0.3)	6.7 (0.2)	6.7 (0.0)	7.0 (0.1)	6.7 (0.1)	<1.0
C10	7.0 (0.2)	7.0 (0.2)	6.9 (0.4)	7.1 (0.2)	7.0 (0.1)	4.3 (0.5)
C11	8.3 (0.1)	8.0 (0.6)	5.9 (0.1)	8.2 (0.2)	5.5 (0.2)	2.0 (0.4)
C12	7.6 (0.1)	7.6 (0.2)	7.4 (0.2)	7.6 (0.3)	7.3 (0.2)	4.0 (0.1)
C13	8.0 (0.5)	7.9 (0.3)	8.1 (0.1)	8.0 (0.1)	7.7 (0.3)	3.4 (0.4)
C14	7.8 (0.1)	7.8 (0.2)	6.7 (0.3)	6.7 (0.2)	6.6 (0.0)	<1.0
C15	7.4 (0.2)	7.6 (0.2)	7.5 (0.3)	7.6 (0.1)	7.2 (0.1)	2.6 (0.2)
C16	8.4 (0.2)	7.7 (0.1)	7.5 (0.0)	8.8 (0.2)	8.3 (0.3)	4.3 (0.2)
C17	7.7 (0.0)	7.2 (0.2)	7.0 (0.0)	5.4 (0.2)	5.4 (0.3)	<1.0
C18	5.8 (0.2)	5.2 (0.2)	5.0 (0.3)	5.4 (0.1)	5.2 (0.2)	<1.0
C19	8.1 (0.1)	6.9 (0.1)	6.2 (0.5)	6.8 (0.2)	6.1 (0.3)	<1.0
C20	6.4 (0.1)	6.3 (0.3)	3.9 (0.2)	6.2 (0.2)	4.5 (0.1)	<1.0

[a] For cheese codes C1–C20, see Table 1; values (log cfu g^{-1}) are means with standard deviation in parentheses.

4.7 log cfu g^{-1} (Caprino di Castel Fiorentino, C2) (Table 4). Total aerobic mesophilic bacteria varied from approximately 4.2 log cfu g^{-1} (Caciocavallo Podolico Dauno, C3) to approximately 8.8 log cfu g^{-1} (Ragusano PDO, C8), with a median value of 7.5 log cfu g^{-1}. The median value for presumptive mesophilic lactobacilli was 7.2 log cfu g^{-1}, with Pecorino Siciliano PDO (C18) and Caprino di Castel Fiorentino (C2) cheeses showing the lowest (approximately 5.2 log cfu g^{-1}) and the highest (approximately 8.7 log cfu g^{-1}) value, respectively. Presumptive thermophilic lactobacilli varied from approximately 3.9 log cfu g^{-1} (Tuma Persa, C20) to approximately 8.1 log cfu g^{-1} (Vaccino, C13), the median value being 6.8 log cfu g^{-1}. Cell density of presumptive mesophilic lactococci ranged from approximately 5.4 log cfu g^{-1} (Maiorchino – C17 and Pecorino Siciliano PDO) to approximately 8.9 log cfu g^{-1} (Caprino di Castel Fiorentino), with a median value of 7.15 log cfu g^{-1}. Presumptive thermophilic streptococci varied from approximately 4.5 log cfu g^{-1} (Tuma Persa) to approximately 8.7 log cfu g^{-1} (Caprino di Castel Fiorentino and Vastedda della valle del Belice PDO, C1), with a median value of 6.75 log cfu g^{-1}.

A number of Gram-positive, catalase-negative, non-motile cocci and rods, able to acidify M17 or MRS broth, were isolated. From each cheese, 15–47 presumptive lactic acid bacteria were considered. All the isolates were assayed for those decarboxylase activities leading to the formation of the four BAs (cadaverine, histamine, putrescine, and tyramine) that exceeded the limit of 100 mg kg^{-1} in at least three cheeses. Arginine-decarboxylase was not considered, since arginine first has to be converted either into ornithine or agmatine, which are direct precursors for putrescine. Tyrosine decarboxylase was the most frequent enzyme activity within isolates, regardless of the group (mesophilic/thermophilic lactobacilli, mesophilic lactococci, and thermophilic streptococci) of lactic acid bacteria (data not shown). Indeed, isolates showing this enzyme activity were found in 16 out of 20 cheeses. The percentage of lactic acid bacteria showing tyrosine decarboxylase activity was 24 or higher for 8 cheeses (Cacio, Caciocavallo Palermitano, Caciocavallo Silano PDO, Canestrato Pugliese PDO, Caprino di Biccari, Maiorchino, Pecorino Foggiano and Pecorino Siciliano PDO). No isolates able to decarboxylate tyrosine were found in Ragusano PDO, Fiore Sicano, Piacentinu Ennese, and Vaccino cheeses. Isolates with histidine decarboxylase activity were found in 15 out of 20 cheeses

and the percentage was high (38%), especially for lactic acid bacteria from Caciocavallo Silano PDO cheese. None of the lactic acid bacteria isolated from Caciocavallo Podolico Dauno, Caprino di Castel Fiorentino, Caprino Girgentano, Maiorchino, and Provola dei Nebrodi cheeses showed histidine decarboxylase activity (data not shown). Cacio, Caciocavallo Podolico Dauno, Cacioricotta, Caprino di Biccari, Caprino Girgentano, Fior di Capra, Pecorino Foggiano, and Vaccino cheeses harboured isolates with lysine decarboxylase activity. Ornithine decarboxylase-positive isolates were found in Cacio, Cacioricotta, Caprino di Biccari, Caprino di Castel Fiorentino, Pecorino Foggiano, Vaccino, and Vastedda della valle del Belice PDO (data not shown). Decarboxylase activities of 20 isolates were confirmed by HPLC analyses (data not shown).

3.4. Correlations between concentrations of biogenic amines and different cheese parameters

PCA was carried out to formulate hypotheses regarding the concentration of the most frequent BAs (cadaverine, histamine, putrescine, and tyramine), as possibly influenced by different cheese parameters. A preliminary PCA revealed that type of milk, thermal treatment of milk/curd, a_w, and cell densities of *Enterobacteriaceae* were not correlated with the concentration of the above mentioned BAs (data not shown). Another PCA was carried out considering the remaining parameters: use of starter culture, type of rennet, cell densities of presumptive lactic acid bacteria and total mesophilic bacteria, concentration of precursor amino acids, percentages of histidine, lysine, ornithine, and tyrosine decarboxylase-positive isolates, time of ripening and pH of the cheese. Two principal components (PC1 and PC2) explained almost 53% of the total variance of the data (Fig. 1). The negative segment of PC1 was related to the cell density of presumptive mesophilic and thermophilic lactobacilli, of mesophilic lactococci, and of thermophilic streptococci, whereas its positive segment was related to the concentration of tyrosine and time of ripening. Concentration of putrescine and tyramine showed the highest loads for PC2. Concentration of lysine and time of cheese ripening showed the highest correlation ($r = \sim 0.87$), followed by cadaverine and tyramine ($r = \sim 0.83$), and by histamine and percentage of histidine decarboxylase-positive bacteria ($r = \sim 0.81$). Histamine
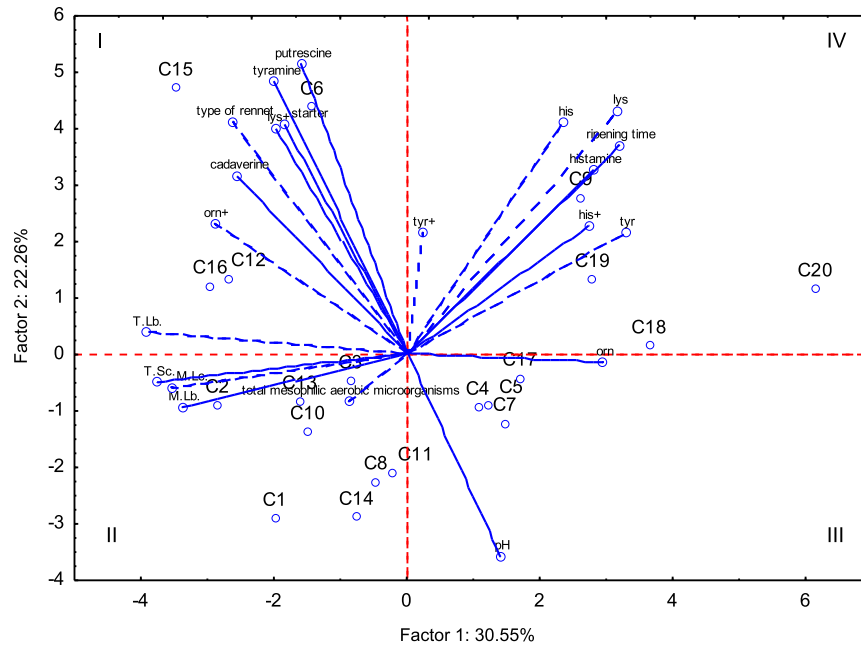
Fig. 1. Score and loading plots of first and second principal components after principal component analysis based on the use of starter (starter), the type of rennet, time of cheese ripening (ripening time), pH, concentrations of histidine (his), lysine (lys), ornithine (orn), tyrosine (tyr), histamine, cadaverine, putrescine, and tyramine, cell densities of total mesophilic aerobic microorganisms, presumptive thermophilic lactobacilli (T.Lb.), mesophilic lactobacilli (M.Lb.), thermophilic streptococci (T.Sc.) and mesophilic lactococci (M.Lc.) of traditional Apulian or Sicilian cheeses, and percentage of histidine decarboxylase- (his+), lysine decarboxylase- (lys+), ornithine decarboxylase- (orn+), and tyrosine decarboxylase- (tyr+) positive lactic acid bacteria isolated from the cheeses. Each cheese is indicated by the alphanumeric code (C1–C20) assigned in Table 1.

also showed relatively high positive correlations with the concentration of histidine ($r = {\sim}0.61$) and time of ripening ($r = {\sim}0.54$). Putrescine was positively correlated with tyramine ($r = 0.76$) and type of rennet ($r = 0.71$). Lower positive correlations were found between each single BA and use of starter ($0.14 < r < 0.44$), as well as between cadaverine and cell density of mesophilic lactococci ($r = 0.43$), and cadaverine and type of rennet ($r = 0.42$). The value of pH and concentration of cadaverine, putrescine, and tyramine were negatively correlated, being the value of r approximately -0.39, -0.40, and -0.29, respectively.

Canestrato Pugliese PDO (C16), Cacio (C12), Caprino di Biccari (C6), and Pecorino Foggiano (C15) cheeses were grouped in the I quadrant mainly because of their high concentration of tyramine and cell density of presumptive lactic acid bacteria. Caciocavallo Silano PDO (C9), Pecorino Siciliano PDO (C18), Piacentinu Ennese (C19), and Tuma Persa (C20) cheeses fell in the IV quadrant, because they were characterised by high concentration of histamine and tyrosine. All the other cheeses were located in the remaining quadrants (II and III) of the plane. These cheeses were characterised by low concentration of BAs, except for Caciocavallo Palermitano (C7) and Fiore Sicano (C11), which contained more than 100 mg of histamine per kg (Fig. 1).

4. Discussion

Consumers' preference for traditional cheeses is currently increasing mainly for two reasons: (i) industrial cheeses are perceived as having "boring" flavour and taste; (ii) consumers prefer the so-called "short distribution chain", probably the main distribution channel for these products. Because the majority of traditional cheeses are subjected to looser controls than industrial ones, their content in BAs may represent a potential safety issue (Ten Brink, Damink, Joosten, & Huis in't Veld, 1990).

In this study, traditional Apulian or Sicilian cheeses were analysed for the concentration of eight BAs, and a number of technological, biochemical and microbiological parameters. Presumptive lactic acid bacteria, isolated from these cheeses, were assayed for their capacity to generate BAs through decarboxylation of precursor amino acids.

In this study, short-ripened (≤ 30 d) cheeses did not show significant BA concentrations. No cheeses showing toxic concentrations of at least one BA were ripened for less than 60 days. PCA showed that BA concentrations and time of ripening were not correlated, except for histamine. Overall, the amount of BAs increases as the time of ripening increases (Bunková et al., 2010; Novella-Rodrìguez, Veciana-Noguès, Trujillo-Mesa, & Vidal-Carou, 2002; Ordóñez, Ibanez, Torre, & Barcina, 1997; Schneller, Good, & Jenny, 1997; Spizzirri et al., 2013). Previous studies (Mayer, Fiechter, & Fischer, 2010; Novella-Rodrìguez, Veciana-Noguès, Izquierdo-Pulido, & Vidal-Carou, 2003) showed that cheeses having similar time of ripening markedly differed from each other in terms of BA concentration, and this was attributed mostly to the extent of ripening. Accordingly, in this study, some cheeses (Ragusano PDO, Fior di Capra, Vaccino) showed low BA concentration, although they were ripened for 90–120 d. Further, time of ripening was highly and positively correlated with concentration of lysine, which may indicate a high extent of proteolysis during ripening.

Within our set of data, histamine was the only BA positively correlated with the corresponding precursor amino acid. Previous studies reported significant positive correlations between BAs and corresponding precursor amino acids (Kebary, El-Sonbaty, & Badawi, 1999; Pinho et al., 2004). On the contrary, no significant correlation was found between tyramine and tyrosine in traditional Turkish Civil cheese (Yildiz et al., 2010). These apparently conflicting results may be explained by the fact that availability of precursor amino acids is not a sufficient prerequisite for BA production, rather than needing the concurrent presence of decarboxylase-positive bacteria is. In this regard, we found a high positive correlation between histamine and percentage of histamine decarboxylase-positive bacteria (Fig. 1). This was in

substantial agreement with a previous study (Schirone et al., 2013). Surprisingly we found that concentration of tyramine, concentration of tyrosine and percentage of tyrosine decarboxylase-positive isolates were not associated (Fig. 1). This could be explained by the ability of some bacterial tyrosine decarboxylases to generate, besides tyramine, 2-phenylethylamine from phenylalanine (Marcobal, De Las Rivas, Landete, Tabera, & Muñoz, 2012). We found that cadaverine was positively correlated with cell density of mesophilic lactococci. Pintado et al. (2008) reported a positive correlation between lactococci and tyramine. Among mesophilic lactococci, some isolates belonging either to *Lactococcus* sp. or *Leuconostoc* sp. were capable of producing cadaverine upon lysine decarboxylation (Marino, Maifreni, Bartolomeoli, & Rondinini, 2008).

Although positive correlation was found between type of rennet and putrescine, based on the results of this study no hypotheses can be done about the role of rennet on BA formation. Indeed, rennet parameters, such as microbiological quality, proteolytic activity, and BA concentration, which would presumably affect BA concentration in cheeses, were not evaluated in this study.

Within our data, PCA showed pH as negatively correlated to the concentrations of tyramine, putrescine, and cadaverine (Fig. 1). Previously, it was shown that low pH (e.g., 4.0–5.5) favours generation of BAs, because FAA decarboxylation is part of microbial cell response to acid stress, which allows the consumption of intracellular protons (Andiç, Gençcelep, Tunçtürk, & Köse, 2010; Fernández et al., 2007; Marcobal, Martin-Alvarez, Moreno-Arribas, & Muñoz, 2006).

We found that starter preparation may affect concentration of BAs. This should not be regarded as surprising, taking into account that some lactic acid bacteria (e.g., *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Streptococcus thermophilus*) used as dairy starters showed the capacity to decarboxylate amino acids precursors of BAs (Burdychova & Komprda, 2007; Marino et al., 2008; Roig-Sagués, Molina, & Hernández-Herrero, 2002). Previous studies provided contradictory results about the role of starters in the production of BAs in cheeses (Marino et al., 2008; Martuscelli et al., 2005). Commercial starters, such as those used for Cacio, Pecorino Foggiano, and Caprino di Biccari, desirably should not contain decarboxylate-positive bacteria. However this feature is often overlooked. Natural starter preparations, such as those used for Caciocavallo Podolico Dauno, Caciocavallo Silano PDO, and Caprino di Castel Fiorentino cheeses, likely harbour bacteria capable of producing BAs.

In this study, Cacio, Canestrato Pugliese PDO, Caprino di Biccari, Pecorino Foggiano, and Piacentinu Ennese cheeses contained at least three BAs at potentially toxic concentrations. Caciocavallo Palermitano, Caciocavallo Silano PDO, Fiore Sicano, Pecorino Siciliano PDO and Tuma Persa cheeses showed food safety issue related to the high concentration of histamine.

5. Conclusions

This study considered all the parameters that potentially affect the concentrations of the four most common BAs in cheese. Unambiguous and ever-valid relations among certain parameters and BAs are difficult to determine. We found that even the time of ripening, always being reported as a key-parameter, cannot be accepted in all cases. This is because BAs are the result of combined and varied factors.

Acknowledgements

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