Comparison of γ-aminobutyric acid and biogenic amine content of different types of ewe's milk cheese produced in Sardinia, Italy

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Abstract

The bioactive compounds γ-aminobutyric acid (GABA) and biogenic amines (BA), together with protein-free amino acids, were measured by high-performance liquid chromatography in ewe's milk cheeses produced in Sardinia with different technological traits. The study included three types of cheese: *Pecorino Sardo* PDO, *Pecorino* and *Casu Marzu*. Farmhouse Casu Marzu and Pecorino showed GABA content (maximum levels: 1001.3 and 378.1 mg 100 g^{-1} respectively) that had never been found so high in cheese before, suggesting that these types of cheese present ideal conditions to produce GABA. These two types of cheese also showed high levels of BA (their total maximum levels were 1035.7 and 288.0 mg 100 g^{-1} respectively). Pearson correlation analysis detected significant correlation between GABA and the main BA present in the cheeses (tyramine, cadaverine and putrescine), suggesting that the factors affecting the production of GABA are the same as those influencing BA formation.

Introduction

Consumers' interest in the relationship between diet and health has increased the demand for information about the specific health characteristics of food. It is well known that food can contain ingredients that may have physiological benefits and/or reduce the risk of chronic disease, or even represent significant risk factors influencing ill health. In cheese, compounds like amino acids are freed during proteolysis; they are substrates for secondary catabolic reactions by means of bacteria with aminoacyl decarboxylase activity that may yield bioactive compounds. Some of them present useful nutraceutical properties, such as γ-aminobutyric acid (GABA), while others, such as biogenic amines (BA), may have negative effects on human health.

Over the last decade attention has been paid to the presence of GABA in cheese and dairy product, as a result of its health-related benefits (Mills *et al.*, 2009; Wang *et al*., 2010; Nejati *et al*., 2013). GABA is a non-protein amino acid acting as one of the main inhibitory neurotransmitters in the sympathetic mammalian nervous system and exerts positive effects in the treatment of sleeplessness, depression, chronic alcohol-related symptoms (Oh *et al*., 2003) and Parkinson's disease (De Jong *et al*., 1984). In addition, it has shown antitumorigenic activity (Thaker *et al*., 2005) and, since it is a strong secregatogue of insulin from the pancreas, it can prevent diabetes (Hagiwara *et al*., 2004).

GABA is produced by the decarboxylation of glutamate catalysed by the enzyme L-glutamic acid decarboxylase (GAD). This enzyme is present in different species of microorganism that could be used for the development of probiotic cheese (Wang *et al.*, 2010). The most interesting are the lactic acid bacteria (LAB), in particular several strains of *Lactobacillus brevis* (Mills *et al.*, 2009), *Lactobacillus plantarum*, *Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus casei* and *Lactococcus lactis* subsp. *Lactis* (Siragusa *et al*., 2007; Mills *et al.*, 2009; Wang *et al*., 2010). The *Enterococcus* spp. and *Streptococcus* spp. also include strains that have shown GAD activity (Hayakawa *et al*., 1997).

Other research has shown that these species of bacteria are also involved in the production of BA (Galgano *et al*., 2001; Linares *et al*., 2012; Lorencová *et al*., 2012), considered as a serious health problem when present at significant levels (Stratton *et al*., 1991). Tyramine, β-phenylethylamine, histamine, tryptamine, cadaverine, putrescine, spermine and spermidine are considered as the most important amines occurring in cheese.

In cheese, BA are considered as an indicator of poor hygienic conditions of raw material and/or manufacturing practices since their production and accumulation is often associated with the activity of contaminant bacteria (Loizzo *et al*., 2013). In general, artisanal sheep cheeses are rich in GABA (Siragusa *et al*., 2007) but also in BA (Loizzo *et al*., 2013; Mascaro *et al*., 2010). In fact, microbial decarboxylase activity requires the availability of free amino acid precursors, produced as an outcome of proteolysis, and favourable pH and temperature conditions that can be achieved during ripening (Siragusa *et al*., 2007; Linares *et al*., 2012; Dhakal *et al*., 2012). Some research has reported that GABA is also present in *Pecorino Sardo* (Siragusa *et al*., 2007), as are BA (Manca *et al*., 2000), even if these studies were limited to a very few samples. For these reasons this study has concerned the simultaneous determination of GABA and BA in Pecorino Sardo produced in cheese factories, *Pecorino* produced in farmhouses, and *Casu Marzu*, made from insect larvae (*Piophila casei*), in order to assess the variability of these nitrogenous compounds important to define cheese quality, in products obtained with different technological traits.

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Materials and Methods

The study was carried out on traditional cheeses made in Sardinia (Italy) from whole ewe's milk. Samples were collected at different dairies and at different stages of ripening. The types of cheese considered were Pecorino Sardo protected designation of origin (PDO), produced at cheese factories (13 samples with ripening time ranging from 30-360 days), farmhouse Pecorino (12 samples with ripening time ranging from 60-360 days), and farmhouse Casu Marzu (9 samples with ripening time ranging from 60-90 days). Pecorino Sardo PDO produced in cheese factories is a semi-cooked cheese made from thermised milk inoculated with a starter culture and coagulated with calf rennet. Ripening takes place in ripening rooms, the temperature (between 6-12°C) and relative humidity (between 80-95%) of which are measured and controlled automatically.

Pecorino cheese obtained from farmhouses is a semi-cooked cheese made from raw milk without a starter culture and coagulated with calf rennet. Ripening takes place in rooms with no control over humidity or temperature, which may even reach room values.

Casu Marzu (also called *casu modde*, *casu cundídu*, or *casu fràzigu* in Sardinian language, which translates *rotten cheese*) is a Sardinian cheese produced with the use of larvae of the cheese fly *Piophila casei*. It is prepared in summer, when higher temperatures favour the life-cycle of the fly, and derived from Pecorino cheese that producers place in the warmer rooms of the plant, uncovered, so that flies have better access to lay their eggs in it. After 60-90 days of ripening the cheese is ready to eat.

To extract the nitrogenous compounds considered, an amount of 1 g of ground cheese was weighed directly in a centrifuge tube and 20 mL of HCl 0.1 M added. The mixture was then homogenised in an ULTRA TURRAX homogeniser (Zipperer, Staufen, Germany) for 5 min. The cheese slurry obtained was centrifuged (ALC PK131R; ALC International S.r.l., Milan, Italy) at 7100 x g for 20 min at 4°C. The supernatant was recovered and the residue re-extracted using the same procedure. The two acid extracts were combined and diluted to 50 mL with HCl 0.1 M. One mL of the extracts was diluted to 10 mL with HCl 0.1 M and then an aliquot of 400 µL was derivatised with Dns-Cl.

To prepare dansyl derivatives of free amino acids (FAA) and BA, the method described by Vinci and Antonelli (2002), was followed, with some modifications. 40 L of saturated sodium carbonate solution at 20°C, 200 L of dansyl chloride solution (1.5% w/v in acetone) and 300 L of acetonitrile were added to 400 L of standard solutions or sample extracts. The vial containing the reaction mixture was capped, vortexed and then incubated at 60°C for 30 min under stirring in a SH 2000-DX Thermo mixer (Finepcr, Seoul, Korea). In order to eliminate the excess of dansyl chloride the mixture was treated with 50 L of L-asparagine solution (2.2% w/v in water), then 10 L of glacial acetic acid was added to remove the excess of carbonate. The solution was filtered through a 0.22 m PVDF syringe filter (Millipore, Bedford, MA, USA) then 10 L were injected in high-performance liquid chromatography.

Simultaneous separation of dansyl derivatives of GABA, protein FAA and BA was performed in a Varian (Walnut Creek, CA, USA) chromatography system equipped with a ProStar 230 Solvent Delivery System, a ProStar 410 autosampler, and an LC 305 fluorescent detector (Linear Instruments, Reno, NV, USA). The system was controlled by Varian Star Chromatography Workstation software (Version 4.5). The column used was an Alltima C_{18} column, 150 mm x 4.6 mm, 3 m [Alltech Italia, Sedriano (MI), Italy], fitted with an Alltima C_{18} guard column, 7.5 mm x 4.6 mm x 5 m, thermostated at 40°C. Detection was carried out with the fluorescent detector operating at 340 and 520 nm as excitation and emission wavelengths respectively.

Free amino acids and BA were determined by the liquid chromatographic method described by Minocha and Long (2004). The solvents used for separation were eluent A: 100% acetonitrile (ACN) and eluent B: 20 mM ammonium acetate buffer (pH 5.9) containing 3% v/v 2-propanol. A gradient programme was implemented as follows: time=0, A:B (10:90), flow=0.5 mL min^{-1}; time=1 min, A:B (10:90), flow=0.5 mL min^{-1}; time=50 min, A:B (65:35), flow=0.5 mL min^{-1}; time=65 min, A:B (100:0), flow=0.5 mL min^{-1}; time=66 min, A:B (100:0), flow=2 mL min^{-1}; time=70 min, A:B (10:90), flow=0.5 mL min^{-1}. An equilibration time of 10 min was applied to achieve mobile phase stabilisation.

The identification of the nitrogenous compounds was performed by comparison of the retention times of peaks in the samples to those of standard solutions and by addition of the suspected compound to the samples. A calibration curve was obtained by analysing standard solutions at 9 different concentrations and calibration graphs were constructed by plotting the peak area versus each analyte concentrations. Quantification was accomplished by direct interpolation in the standard curves for each compound.

Statistical analysis

Statistical analyses of the data were performed using the software package SPSS 14 (SPSS Inc., Chicago, IL, USA). The results were expressed as means of two replications. Pearson correlation analysis was conducted to determine the relationship between the different compounds considered. Statistical significance was declared at P=0.01.

Results and Discussion

On the basis of the original method designed for the analysis of FAA and polyamines (Minocha and Long, 2004), we developed a method that allowed extending the analysis to other seven BA. To do this, it was necessary to extend the chromatographic running time from 56 to 70 min. In Figure 1 the chromatograms of a cheese sample and of a standard solution of amino acids and amines are shown. Dansyl amino acids and dansyl BA were eluited in the following order: aspartic acid, glutamic acid, serine, threonine, glycine, alanine, arginine, proline, γ-amino butyric acid, valine, methionine, isoleucine, leucine, phenylalanine, ornithine, cysteine+cystine, lysine, histidine, tyrosine, agmatine, tryptamine, phenylethylamine, putrescine, cadaverine, hystamine, serotonin, tiramine, spermidine and spermine.

The content of the compounds analysed in the three types of ewe's milk cheese considered are shown in Table 1. The highest level of GABA was found in Casu Marzu (ranging from 34.4 to 1001.3 mg 100 g^{-1}) and in Pecorino

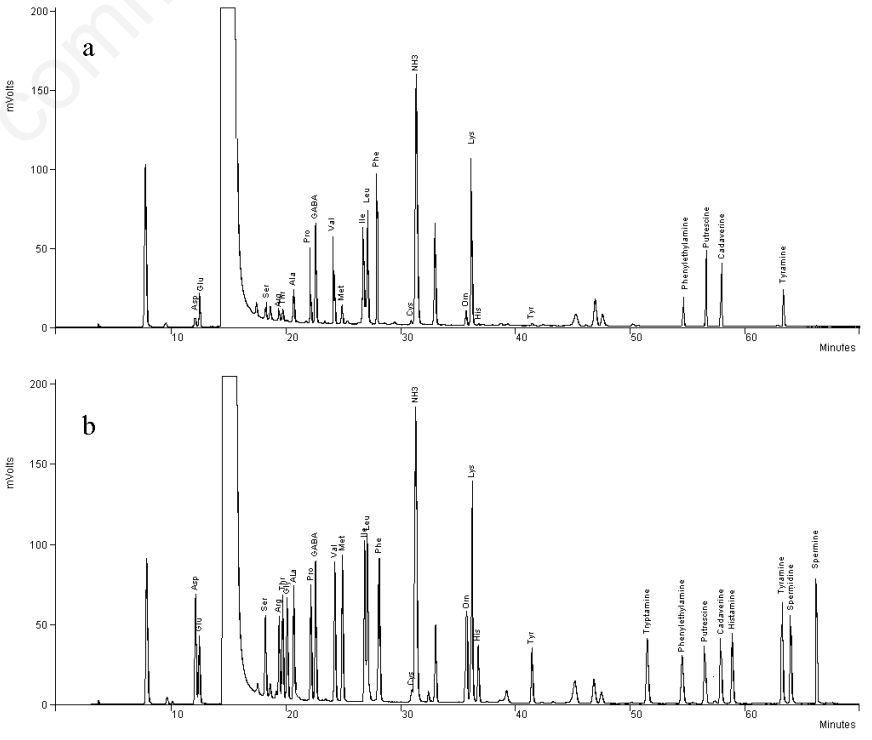

Figure 1. Chromatogram of a cheese sample (a) and of a standard solution of amino acids and amines (b).

(ranging from 0.0 to 378.1 mg 100g^{-1}), while the samples of Pecorino Sardo PDO showed a lower content (ranging from 2.3 to 52.0 mg 100g^{-1}). The results showed great variability in GABA content not only between the three types of cheese but also within the same type. By comparing these Sardinian products with other ewe's milk cheeses it was noted that in most of the samples of both Casu Marzu and Pecorino the GABA content was from 2 to 10 times higher than the maximum level (39.1 mg 100g^{-1}) measured in other types of Italian cheese (Siragusa *et al*., 2007). In particular, three samples of Casu Marzu had a content that was as much as 20 times greater than that reported in the above-mentioned work. The differences in GABA content appear to be dependent on the different degree of proteolysis observed between the three types of cheese. In fact, considering the content of total FAA as an index of proteolysis level, calculated as the sum of the individual FAA measured, a significant correlation with GABA was found (Pearson coefficient 0.555 at P=0.01). As for GABA, the higher levels of Total FAA were found in the samples of Casu Marzu. This is most probably due to the high proteolytic activity induced by the *Piophila casei* larvae, and to the high temperature used for cheese storage (Mazzette *et al*., 2010). The farmhouse samples of Pecorino tended to have a Total FAA content greater than those of Pecorino Sardo PDO from cheese factories. As reported in the literature, such differences can be attributed to changes in manufacturing processes (Pintado *et al*., 2008; Schirone *et al*., 2011). By comparison, in cheese samples with the same age, great variability in Total FAA content was also found within the same type of cheese, highlighting that production processes can vary from one cheesemaker to another. In Pecorino Sardo, as observed in a previous work (Manca *et al*., 1999), variability in the FAA content was due to a production protocol that allowed the use of different technological features. Considering the amino acid profile (percentage of the Total FAA for each amino acid) shown in Table 2, it is possible to highlight that in three samples of Pecorino and in three of Casu Marzu, GABA is the amino acid present in the highest percentage with respect to the Total FAA (from 12.3 to 18.5 and from 14.2 to 22.9% respectively). In almost all the other samples, including the factory products, glutamic acid, the precursor of GABA, is the amino acid mostly represented, followed by leucine, valine and lysine. This FAA profile is similar to that found in other ewe's milk cheese (Izco *et al*., 2000; Barcina *et al*., 1995), while to our knowledge, GABA has never been found as the major FAA in sheep's cheese.

As for GABA and Total FAA, great variability in the total content of BA was found between the different types of cheese, but also within the same type. Casu Marzu showed the highest content of Total BA; in particular, two samples presented values of 1035.7 and 923.0 mg 100 g^{-1} respectively. The other Casu Marzu samples had values ranging from 62.9 to 282.1 mg 100 g^{-1} for this parameter, more similar to that found in other artisanal Italian cheeses, such as Formaggio di Fossa (Mascaro *et al*., 2010)

Table 1. Content of γ-aminobutyric acid, total free amino acids, and biogenic amines in different types of ewe's milk cheese produced in Sardinia (mg 100 g^{-1}).

Types of cheese	Ripening time (days)	GABA	Total FAA	Triptamine	β-phenyl-ethylamine	Putrescine	Cadaverine	Histamine	Tyramine	Spermidine	Spermine	Total BA
Pecorino Sardo PDO	30	2.5	281.3	1.6	nd	0.1	1.3	nd	1.6	1.0	0.9	6.5
	30	2.3	334.7	nd	nd	0.1	1.4	0.8	3.5	nd	4.0	9.8
	60	4.5	822.9	9.3	nd	1.1	1.4	nd	19.3	0.6	1.8	33.5
	60	3.7	815.0	5.7	nd	0.1	1.4	0.5	3.5	2.9	0.4	14.4
	60	4.2	768.7	2.1	nd	0.1	9.7	7.2	3.5	1.1	0.4	24.2
	60	3.6	794.6	1.4	nd	1.1	1.7	0.7	1nd	nd	5.4	20.2
	90	8.8	1473.0	2.4	nd	0.7	0.9	2.7	4.4	7.2	1.0	19.2
	150	2.7	1615.6	3.2	nd	0.1	3.6	2.1	7.9	3.4	1.0	21.3
	150	13.7	578.7	nd	nd	nd	0.1	nd	1.4	2.7	0.2	4.4
	180	2.8	588.1	1.7	nd	0.5	0.3	3.3	1.0	0.4	0.3	7.4
	180	6.1	934.4	nd	nd	0.1	1.3	0.6	0.4	0.6	0.2	3.1
	270	52.0	1387.0	nd	nd	nd	0.7	4.3	6.4	11.1	1.2	23.6
	360	10.2	1893.2	nd	nd	0.8	2.6	5.3	16.3	0.5	2.4	27.8
Pecorino	60	71.3	619.3	nd	0.9	3.4	15.4	nd	5.7	nd	nd	25.5
	60	20.6	840.8	2.9	2.1	1.1	5.3	nd	4.7	nd	nd	16.1
	90	228.4	1438.3	nd	nd	2.7	11.0	nd	30.8	nd	nd	44.5
	90	378.1	2046.7	4.1	8.6	92.7	137.0	24.4	21.0	nd	nd	287.8
	150	nd	1242.4	nd	nd	nd	nd	nd	13.2	nd	nd	13.2
	150	11.6	1480.5	nd	nd	16.4	5.5	nd	37.3	nd	nd	59.2
	180	20.5	3266.5	nd	5.4	nd	4.8	nd	68.6	nd	nd	73.4
	180	135.4	5401.8	11.1	4.7	13.0	1.7	128.4	93.0	nd	nd	247.2
	180	9.8	411.1	nd	nd	nd	21.3	19.5	1.6	nd	nd	42.4
	270	133.3	1973.9	nd	nd	nd	5.2	nd	4.2	nd	nd	9.4
	360	339.0	2754.4	5.4	3.0	33.6	33.2	33.4	17.8	nd	nd	126.4
	360	75.3	5268.9	13.5	7.1	5.1	33.6	nd	19.7	nd	nd	79.0
Casu Marzu	60	51.3	2393.4	nd	4.9	4.1	28.8	nd	3nd	nd	nd	62.9
	60	203.5	1946.6	5.1	nd	1.9	3.1	nd	61.0	nd	nd	71.1
	90	122.5	4019.7	nd	10.8	67.8	8.8	nd	150.1	nd	nd	226.7
	90	63.2	4344.5	16.0	16.2	90.6	8.0	nd	167.5	nd	nd	282.1
	90	1001.3	7051.6	15.8	13.2	58.2	69.6	nd	93.7	nd	nd	237.3
	90	66.4	6319.9	12.0	10.8	39.1	6.3	nd	187.3	nd	nd	244.7
	90	959.8	4196.8	33.2	90.9	140.3	462.0	99.3	188.2	nd	nd	923.0
	90	34.4	2535.8	nd	nd	8.3	24.7	35.6	47.1	nd	nd	115.7
	90	806.2	3705.5	41.8	nd	165.8	470.7	126	231.4	nd	nd	1035.7

GABA, γ-aminobutyric acid; FAA, free amino acids; BA, biogenic amine; PDO; protected designation of origin; nd, not detected.

and Pecorino di Farindola (Schirone *et al*., 2011). Considering that threshold values of 75-90 mg 100g^{-1} have been proposed for Total BA in food (Spanjer and Van Roode, 1991; ten Brink *et al*., 1990), Casu Marzu can be considered unsafe, especially for patients treated with monoamine oxidase inhibitor drugs (MAOIs). Also four samples of Pecorino showed a Total BA content (between 79.0 and 287.8 mg 100 g^{-1}) that exceeded the threshold proposed, while the other samples presented values ranging from 9.4 and 73.4 mg 100 g^{-1} that did not represent a risk for consumers' health. However, the samples of Pecorino had, in general, a higher level of Total BA than those of Pecorino Sardo PDO, whose values varied from 3.1 to 33.5 mg 100 g^{-1}. Seeing that BA are considered an index of poor hygienic conditions, the high content of these compounds measured in Casu Marzu and Pecorino could be related to less than optimal environmental conditions during the production process (Mazzette *et al*., 2010). The use of insects, as in Casu Marzu, and of raw milk, combined with a high storage temperature, seems to favour the production of BA, as already shown in other artisanal sheep's cheeses (Loizzo *et al*., 2013; Mascaro *et al*., 2010). On the contrary, in Pecorino Sardo PDO, where the milk used is thermised and the temperature and humidity conditions under control, the products showed a low content of total BA. The variability observed in BA content, as observed by Novella-Rodriguez *et al*. (2003) and Pintado *et al*. (2008) in other types of cheeses, could be attributable to differences in the manufacturing process. A positive correlation was found between total BA and total FAA (Pearson coefficient=0.513 at P=0.01), confirming the findings of Giraffa *et al*. (1995) who observed an increase in BA corresponding to an increase in proteolysis. Tyramine, cadaverine and putrescine were the main amines in all the types of cheese considered, even if their content varied to a great extent: in this respect the statements made previously are valid for the total content of BA. In Casu Marzu the highest levels found for tyramine, cadaverine and putrescine were 231.4, 470.7 and 165.8 mg 100 g^{-1} respectively. Therefore, by comparison with the literature (Loizzo *et al*., 2013; Schirone *et al*., 2013), some samples of Casu Marzu seem to present the highest contents found in cheeses up to now. In blue cheese –

Table 2. Free amino acid profile of the different types of ewe's milk cheese produced in Sardinia (percentage of the total free amino acids for each amino acid).

Types of cheese	Ripening time (days)	Asp	Glu	Ser	Thr	Gly	Ala	Arg	Pro	GABA	Val	Met	Ile	Leu	Phe	Orn	Cys+ cystine	Lys	His	Tyr
Pecorino Sardo PDO	30	2.1	8.4	1.7	0.4	1.2	5.6	21.3	6.5	0.9	9.5	5.0	3.8	10.1	8.8	1.3	0.1	9.3	0.9	3.1
	30	0.2	18.9	3.2	0.5	1.3	9.7	9.2	5.0	0.7	1nd	3.3	3.0	10.2	7.9	1.5	0.1	9.7	1.6	4.3
	60	2.0	18.9	1.1	0.1	0.7	9.8	6.1	2.9	0.5	10.8	1.7	1.9	17.1	10.5	1.2	nd	5.7	5.8	3.1
	60	1.1	16.5	2.1	2.9	1.6	6.0	6.8	7.3	0.5	9.6	4.0	4.1	11.9	7.6	1.7	nd	11.8	2.3	2.2
	60	3.9	22.1	2.4	2.9	1.1	4.6	1.7	8.2	0.5	9.7	3.7	4.3	12.2	6.9	2.2	0.1	8.0	2.6	2.7
	60	6.2	22.4	1.8	0.6	0.7	6.0	7.7	4.1	0.5	9.5	3.6	2.6	10.9	7.6	1.6	0.1	9.3	1.6	3.3
	90	4.9	18.4	3.1	3.5	1.5	4.2	nd	8.6	0.6	8.4	3.7	5.1	10.2	6.5	1.9	nd	13.2	4.0	2.2
	150	6.9	21.0	nd	1.1	0.7	5.1	7.2	5.6	0.2	8.6	3.4	4.0	10.7	6.3	1.5	nd	12.0	3.1	2.5
	150	2.0	5.4	1.6	1.1	1.5	4.9	5.7	6.7	2.4	14.2	5.3	3.9	14.9	12.1	1.5	nd	11.8	1.7	3.2
	180	2.6	17.2	3.1	2.2	1.6	4.1	0.1	8.5	0.5	8.9	3.8	6.3	10.7	6.6	2.0	0.1	14.5	4.0	3.1
	180	7.3	25.5	4.3	2.5	1.6	4.4	nd	7.3	0.7	6.4	2.5	5.6	7.6	4.3	3.4	0.1	10.4	4.0	2.1
	270	7.0	2.1	1.9	0.4	0.6	1.6	10.4	12.1	3.7	11.6	4.2	7.5	13.2	7.4	1.3	nd	9.5	0.1	5.2
	360	6.4	9.1	2.4	3.5	1.8	4.9	5.2	9.6	0.5	8.3	4.0	0.7	11.1	6.4	3.1	2.9	14.1	5.0	1.0
Pecorino	60	9.2	9.0	nd	1.9	nd	5.9	1.9	3.7	11.5	12.3	5.6	5.7	13.3	7.7	3.3	nd	9.2	nd	nd
	60	7.1	27.5	nd	2.2	nd	4.3	2.3	4.3	2.5	9.9	5.2	6.5	12.3	nd	3.9	2.4	9.6	nd	nd
	90	4.8	2.0	3.8	3.2	1.7	5.4	nd	6.0	15.9	9.4	4.8	6.3	13.2	7.7	1.1	nd	12.4	nd	nd
	90	0.9	1.9	nd	3.4	nd	10.2	2.4	9.1	18.5	13.1	5.5	9.1	16.6	5.5	1.1	nd	2.8	nd	nd
	150	nd	20.3	nd	nd	0.9	4.1	nd	4.2	nd	13.4	3.8	3.0	18.0	10.8	6.0	nd	10.6	nd	3.2
	150	9.6	24.8	nd	1.2	1.1	2.6	nd	3.1	0.8	10.5	2.9	4.0	16.8	8.4	1.1	nd	9.1	nd	1.8
	180	4.1	17.9	3.6	2.5	1.9	3.9	nd	7.7	0.6	9.2	2.9	6.5	11.1	6.3	4.7	nd	11.7	3.0	0.9
	180	5.7	16.8	nd	2.2	2.1	4.2	nd	11.8	2.5	8.7	3.3	6.5	10.2	5.5	5.1	nd	12.5	nd	1.0
	180	0.6	25.6	nd	0.8	nd	7.0	1.2	7.1	2.4	14.6	3.9	7.9	16.8	nd	2.1	nd	10.1	nd	nd
	270	3.9	21.0	1.7	2.4	nd	5.0	3.4	6.0	6.8	9.5	4.2	6.7	11.7	4.1	3.9	nd	9.8	nd	nd
	360	5.1	12.5	2.4	5.9	nd	4.3	2.4	10.9	12.3	9.6	2.6	9.0	9.0	nd	0.5	3.5	1nd	nd	nd
	360	5.9	23.7	1.5	3.8	nd	4.3	3.3	7.8	1.4	9.4	4.2	7.8	9.3	3.8	1.9	nd	10.1	1.7	nd
Casu Marzu	60	4.7	14.1	1.0	2.2	0.6	6.0	nd	3.0	2.1	8.0	5.0	6.7	12.0	6.7	4.2	nd	13.0	4.2	3.0
	60	1.9	6.6	2.2	2.1	1.3	6.0	nd	6.5	10.5	10.1	4.4	5.6	16.8	8.2	4.1	nd	9.6	nd	2.1
	90	2.7	15.4	nd	2.8	0.8	5.3	nd	9.4	3.0	8.6	4.4	5.9	11.3	6.9	1.8	nd	13.5	3.2	1.2
	90	1.9	13.0	1.0	1.9	1.1	5.0	nd	9.0	1.5	9.5	4.5	6.3	11.9	6.0	2.5	nd	16.0	2.3	1.8
	90	2.3	9.0	1.1	1.1	3.7	4.5	nd	11.8	14.2	9.7	2.8	6.7	10.8	5.9	0.6	nd	10.4	1.5	1.4
	90	3.6	14.6	2.9	3.2	1.7	4.0	nd	10.7	1.1	8.1	4.0	6.3	11.9	6.8	2.6	nd	12.6	2.5	1.4
	90	nd	2.2	0.9	1.7	2.1	12.1	nd	8.7	22.9	10.8	4.1	8.4	15.7	5.1	0.5	nd	2.5	nd	nd
	90	3.7	14.5	1.7	1.9	0.7	5.3	nd	3.4	1.4	5.8	5.0	5.0	12.8	6.2	4.1	nd	10.5	5.3	3.9
	90	nd	1.4	0.5	1.4	1.9	11.9	nd	6.8	21.8	10.3	5.0	8.9	18.1	5.9	0.2	nd	0.6	nd	nd

Asp, aspartic acid; Glu, glutamic acid; Ser, serine; Thr, threonine; Gly, glycine; Ala, alanine; Arg, arginine; Pro, proline; GABA, γ-aminobutyric acid; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Orn, ornithine; Cys+ Cys, cysteine; Lys, lysine; His, histidine; Tyr, tyrosine. PDO, protected designation of origin; nd, not detected.

Table 3. Pearson correlation coefficients among the variables γ-aminobutyric acid, total free amino acids and biogenic amines measured in different types of ewe's milk cheese produced in Sardinia.

	GABA	Triptamine	Putrescine	Cadaverine	Histamine	Tiramine	Spermidine	Spermine	β-phenyl-ethylamine	TOT FAA	TOT BA
GABA	1	0.763**	0.779**	0.795**	0.557**	0.595**	-0.182	-0.225	0.610**	0.555**	0.794**
Triptamine	0.763**	1	0.837**	0.859**	0.706**	0.780**	-0.185	-0.140	0.572**	0.568**	0.513**
Putrescine	0.779**	0.837**	1	0.857**	0.616**	0.824**	-0.207	-0.229	0.603**	0.519**	0.917**
Cadaverine	0.795**	0.859**	0.857**	1	0.728**	0.638**	-0.142	-0.156	0.651**	0.301	0.938**
Histamine	0.557**	0.706**	0.616**	0.728**	1	0.553**	-0.141	-0.162	0.413*	0.367*	0.947**
Tiramine	0.595**	0.780**	0.824**	0.638**	0.553**	1	-0.236	-0.282	0.533**	0.731**	0.783**
Spermidine	-0.182	-0.185	-0.207	-0.142	-0.141	-0.236	1	0.094	-0.134	-0.252	0.836**
Spermine	-0.225	-0.140	-0.229	-0.156	-0.162	-0.282	0.094	1	-0.149	-0.260	-0.189
β-phenyl-ethylamine	0.610**	0.572**	0.603**	0.651**	0.413*	0.533**	-0.134	-0.149	1	0.402*	-0.217
Total FAA	0.555**	0.568**	0.519**	0.301	0.367*	0.731**	-0.252	-0.260	0.402*	1	0.648**
Total BA	0.794**	0.917**	0.938**	0.947**	0.783**	0.836**	-0.189	-0.217	-0.217	0.513**	1

GABA, γ-aminobutyric acid; FAA, free amino acids; BA, biogenic amine. *Correlation is significant at the P<0.05; **correlation is significant at P<0.01.

one of the richest in BA (Loizzo *et al*., 2013; Novella-Rodriguez *et al*., 2003) – the highest levels of tyramine, cadaverine and putrescine were 158.5, 210.4 and 25.7 mg 100 g^{-1} respectively. Regardless of the type of cheese, histamine, triptamine and β-phenylethylamine were present in only about 50% of the samples and also for these amines the highest concentrations were found in samples of Casu Marzu and Pecorino. The polyamines spermidine and spermine were present only in Pecorino Sardo PDO, with a level ranging from 0.0 to 11.1 and from 0.0 to 5.4 mg 100 g^{-1} respectively, similar to that found in blue cheese (Novella-Rodriguez *et al*., 2003). Among the amines determined, agmatine and serotonin were never found. This is in accordance with previous works in which these amines are usually present at low levels or are not detected in cheese (Loizzo *et al*., 2013; Custodio *et al*., 2007; Novella-Rodriguez *et al*., 2003).

Relationship between γ-aminobutyric acid and biogenic amines

The Pearson test showed a significant correlation (P=0.01) between GABA and the amines putrescine, cadaverine and triptamine (Pearson correlation coefficients 0.779, 0.795 and 0.763 respectively) (Table 3). Therefore, it may be assumed that factors responsible for BA production, such as temperature, pH, availability of FAA and, primarily, microorganisms with amino acid decarboxylase activity (Pinho *et al*., 2001), may also affect GABA synthesis (Siragusa *et al*., 2007; Dhakal *et al*., 2012).

The literature highlights that in cheese and other dairy products some microbial species responsible for the synthesis of one or more BA are also involved in GABA production. In particular, the genera *Lactobacilli* include several strains of the species *Lb. paracasei, Lb. delbrueckii, Lb. plantarum, Lb. brevis,* and *Lactococcus lactis,* active in GABA production (Siragusa *et al*., 2007) but some of them can form one or more BA (Linares *et al*., 2012; Galgano *et al*., 2001); likewise a *Streptococcus thermophilus* strain can form GABA, tyramine and histamine. Cadaverine, putrescine and histamine (Maifreni *et al*., 2013), but also GABA could be produced by species belonging to the genera *Enterococcus* (Dhakal *et al*., 2012). Many of these species of bacteria, in particular *Lb. brevis*, *Lb. plantarum*, *Lb. delbrueckii*, *Lb. paracasei*, *Lactococcus lactis, Streptococcus thermophilus* and *Enterococcus spp*, were found as components of the microflora of traditional Pecorino Sardo cheese (Mannu *et al*., 2002; Madrau *et al*., 2006). Therefore, the types of sheep's cheese considered seem to present the ideal conditions for favouring the production and accumulation of both GABA and BA.

Conclusions

Great differences were found for GABA, total FAA and BA content in the three types of cheese considered. Farmhouse Casu Marzu and Pecorino are the types of cheese with a high content of GABA, a compound with beneficial functions, but also of BA, in particular tyramine, cadaverine and putrescine that in high concentrations represent a health hazard for consumers. The Pearson correlation test revealed that variability in the content of GABA and BA was correlated with the total FAA, indicating that their production depends on the intensity of the proteolytic phenomena and thus on environmental and manufacturing factors that affect this biochemical event.

Given the significant correlation highlighted by the Pearson test (P=0.01) between GABA and the most frequently represented BA, it was supposed that the production of all these compounds is affected by the same factors. Furthermore, it was shown that the cheeses considered, in particular those of farmhouse Casu Marzu and Pecorino, presented ideal conditions for developing microorganisms and for their carboxylase activity. Considering the interest of the food industry in functional food, Casu Marzu and Pecorino microflora can potentially be useful to enrich cheese or milk-fermented products with GABA, but further studies are needed to assess whether microbial strains are present that are capable of producing a high content of GABA but not of BA and to individuate which technological factors may favour the grows of these bacteria.

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