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Qualitative and quantitative evaluation of biogenic amines *in vitro* production by bacteria isolated from ewes' milk cheeses

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Complete List of Authors:	Torracca, Beatrice; University of Pisa, Department of Veterinary Sciences Pedonese, Francesca; University of Pisa, Department of Veterinary Sciences Turchi, Barbara; University of Pisa, Department of Veterinary Sciences Fratini, Filippo; University of Pisa, Department of Veterinary Sciences Nuvoloni, Roberta; University of Pisa, Department of Veterinary Sciences
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Qualitative and quantitative evaluation of biogenic amines *in vitro* production by bacteria isolated from ewes' milk cheeses

Running title: Biogenic amines production by bacteria isolated from ewes milk cheeses

Torracca Beatrice[1], Pedonese Francesca[1], Turchi Barbara[1], Fratini Filippo[1], Nuvoloni Roberta[1]

[1]Department of Veterinary Science, University of Pisa, Pisa, Italy

Corresponding author: Beatrice Torracca, Department of Veterinary Science, University of Pisa, Viale delle Piagge 2, 56124 Pisa. beatrice.torracca@for.unipi.it tel +390502216960

ORCID IDs

Beatrice Torracca – orcid.org/0000-0001-9233-4136

Francesca Pedonese – orcid.org/0000-0002-4320-4770

Barbara Turchi – orcid.org/0000-0002-7969-2489

Filippo Fratini – orcid.org/0000-0002-1717-2947

Roberta Nuvoloni – orcid.org/0000-0002-7469-571X

Abstract

Studying the production of biogenic amines (BA) by cheese microbiota is important, because high BA concentrations in food represent a health risk for consumers. Qualitative screening with differential media and HPLC quantification were used to investigate the production of 8 BA (2-phenylethylamine, cadaverine, histamine, putrescine, spermidine, spermine, tryptamine, tyramine) by 72 isolates from curd and cheese samples manufactured with raw and pasteurised ewes' milk. *Enterobacteriaceae* showed good putrescine and cadaverine production, both for number of positive isolates and for concentrations produced (average: 341 and 785 µg ml^{-1} , respectively). Among *Enterobacteriaceae* data are provided on BA formation by *Pantoea conspicua*, previously not isolated from food samples. All enterococci formed tyramine, often in high amounts (average: 1608 µg ml^{-1}), and many produced notable 2-phenylethylamine, putrescine and cadaverine concentrations (average: 184, 121 and 146 µg ml^{-1}, respectively). BA formation by lactobacilli was overall extremely limited, with the notable exception of high tyramine concentrations produced by 1 *Lactobacillus paracasei* (800 µg ml^{-1}) and 2 *Lactobacillus curvatus* (> 1700 µg ml^{-1}), all isolated from pasteurised milk samples. Thus, undesired and technologically useful microorganisms both play a role in BA accumulation in cheeses.

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Keywords: biogenic amines, tyramine, HPLC, enterococci, lactobacilli, ewes' milk cheese

Introduction

The formation and accumulation of biogenic amines (BA) in food has been widely studied, because, at high concentrations, they can pose a health risk for consumers [1]. The clinical symptoms and their severity vary with the amount and variety of BA ingested and also with the detoxificating activity in the gut, which itself varies from individual to individual and it is influenced by certain drugs or alcohol consumption, among others [2]. In particular, histamine (HI) and tyramine (TY) can have important psychoactive or vasoactive effects in humans and are responsible, respectively, for the food diseases known as "scombroid poisoning" and "cheese reaction" [3]. Moreover, secondary amines, like putrescine (PUT) and cadaverine (CAD) can react with nitrites in food, forming carcinogenic nitrosamines [4]. In Europe, only the amount of HI in certain families of fish is regulated [5], although recent studies on the *in vitro* cytotoxicity of HI and TY suggest that TY cytotoxicity is higher than that of HI [6].

In food, including cheeses, BA are usually the result of bacteria's metabolism, and are produced by the decarboxylation of precursor amino acids. In order to study BA production by microorganisms, molecular technologies can be employed to detect the presence of genes encoding for specific decarboxylases within the bacterial genome. Furthermore, several screening procedures based on differential cultural media have been developed and employed to detect effective BA production *in vitro* by microorganisms [7], while BA production quantification is usually performed with High Performance Liquid Chromatography (HPLC) [8]. For food safety, it is important to study the capacity of BA formation by the different bacterial groups, both to understand BA accumulation due to natural or contaminant microflora, and for the selection of microorganisms to employ in technological processes. Such studies must be carried out at the strain level because, within the same species, a great variation has been reported in the type and quantity of BA produced [1].

In cheese, lactic acid bacteria (LAB), and enterococci in particular, are known to produce TY, PUT and HI, possibly in high amounts [9-10]. *Enterobacteriaceae* are known to produce HI, and possibly high levels of CAD and PUT [4, 11], and for this reason the presence of CAD and PUT in cheeses has been proposed as an indicator of food hygiene, especially considering that ornithine decarboxylase positive, and thus PUT-producing, LAB strains are not of dairy relevance [12].

Following a previous study on BA content in cheeses manufactured in Tuscany [13] in which high levels of BA were found, the aim of this work was to investigate the microorganisms responsible for BA accumulation in such cheeses, evaluating the production of 8 BA (2-phenylethylamine (PHN), CAD, HI, PUT, spermidine (SPD), spermine (SPM), tryptamine (TRN), TY) by bacteria isolated from curd and cheese samples produced with ewes' milk.

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Materials and Methods

Isolation and purification of potential BA producing bacteria

Potential decarboxylase positive bacteria (*Enterobacteriaceae,* enterococci, lactobacilli) were isolated from curd and cheese samples collected during a study on BA content in cheeses manufactured in Tuscany with pasteurised and raw ewes' milk [13]. The studied microbial groups were not part of the starter cultures employed for cheese production. After sample dilution with 2% (w/v) sterile sodium citrate solution, *Enterobacteriaceae* were isolated on Violet Red Bile Glucose Agar (VRBGA) after 24 h incubation at 37 °C, enterococci were isolated on Kanamycin Aesculin Azide Agar base with kanamycin selective supplement after incubation at 42 °C for 48 h, and lactobacilli were isolated on de Man, Rogosa, and Sharpe (MRS) agar after 72 h incubation in anaerobic jars at 30 °C. All culture media and supplements were purchased from Oxoid Ltd. (Basingstoke, UK). Selected colonies were purified by streaking them, using the aforementioned media and incubation conditions, at least three times.

Seventy two isolates were selected to be tested for BA production, such as to reflect the relative presence of the microbial groups in the studied samples. The number of tested isolates for each microbial group, and the type of samples they came from is detailed in Table 1.

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Identification of the isolates

Enterobacteriaceae were identified with API 20 E kit (bioMérieux, Marcy-l'Étoile, France) and the correlated Apiweb Stand Alone v.1.1.0 software; when identification parameters were not reliable (%ID < 95% and/or T < 0.6) 16S rRNA sequencing was performed (see below). Enterococci were identified with species-specific PCR for *Enterococcus faecalis* and *Enterococcus faecium,* according to Dutka-Malen et al. [14], after DNA extraction was performed using the GenElute Bacterial Genomic DNA kit (Sigma Aldrich Inc., Saint Louis, MO, USA) following manufacturer's instruction. Lactobacilli were preliminarily identified using the API 50 CH kit with the CHL medium (bioMérieux, Marcy-l'Etoile, France) and confirmed with species-specific PCR (*Lactobacillus curvatus* and *Lactobacillus plantarum* following Berthier and Ehrlich [15], *Lactobacillus fermentum* following Dickson et al. [16], *Lactobacillus paracasei* and *Lactobacillus rhamnosus* following Desai et al. [17]), after DNA extraction as described above. When identification was not confirmed, 16S rRNA sequencing was performed.

For the 16S rRNA sequencing, after DNA extraction as previously described, the protocol of Marchesi et al. [18] was used to amplify a gene sequence of approximately 1030 bp; the amplicons were sent out (BMR Genomics, Padoa, Italy) to be subjected to Sanger sequencing. The obtained sequences were identified evaluating the closest relatives in the

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GenBank database using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) from the National Center of Biotechnology Information (NCBI).

Qualitative screening of BA production

Enterococci and *Enterobacteriaceae* isolates were cultured in Tryptone Soy Broth (24 h, 37 °C) and lactobacilli in MRS Broth (24 h, 37 °C). Each tested isolate was then inoculated at 0.1 % (v/v) in 8 tubes of decarboxylase screening broth, one as is and 7 enriched at a rate of 1 % w/v with one of the amino acid precursors (L-arginine hydrochloride, L-histidine hydrochloride monohydrate, L-lysine hydrochloride, L-ornithine hydrochloride L-phenylalanine, L-tyrosine disodium salt monohydrate; Sigma Aldrich srl, Milano, Italy) of the studied amines. One test was carried out with the addition of arginine, since PUT can be produced both by direct decarboxylation of ornithine, and also indirectly from the amino acid arginine or its corresponding amine agmatine. As a precursor of tyramine, the disodium salt of tyrosine was used to overcome the poor solubility of tyrosine, as suggested by Bover-Cid and Holzapfel [7]. For enterococci and *Enterobacteriaceae,* Moeller Decarboxylase Broth [19] was used as decarboxylase screening broth, while for lactobacilli the differential media described by Bover-Cid and Holzapfel [7] was used, but in a not agarised form. In all cases, the final pH of the broth was adjusted to 5.3. Inoculated tubes were incubated at 37 °C for 72 h, and isolates were considered decarboxylase positive if the media turned purple in the absence of a positive reaction in the corresponding control tube. All culture media were acquired from Oxoid Ltd. (Basingstoke, UK).

Quantitative determination of *in vitro* BA production by HPLC analysis

For the quantification of BA production, isolates were cultured and inoculated as described for the qualitative screening, except that, for each isolate, one single tube was used, enriched with all the 7 amino acid precursors (1% w/v each). After incubation at 37 °C for 72 h, broths were centrifuged (3000 g, 5 minutes) and the supernatant was filtered through a nylon 0.45 µm syringe filter. BA extraction, dansyl chloride derivatization and HPLC analyses were performed as detailed in Torracca et al. [20], following a procedure based on the one described by Innocente et al. [21] with some modifications. Briefly, 8 ml of 0.1 M HCl were added to 2 ml of the filtered sample, followed by the addition of 20 µl of 1,7 diaminoheptane (10 mg ml^{-1}) (Sigma Aldrich Srl, Milan, Italy), used as internal standard. After the addition of 5 ml of saturated $NaHCO_3$ solution, the pH of the extract was adjusted to 11.5 using 5 M NaOH. For the derivatization, 2 ml of dansyl chloride (5 mg ml^{-1} in acetone) were added to 3 ml of the sample and then incubated 60 minutes at 40 °C. Then 400 µl of L-proline (100 mg ml^{-1}) were added and the sample was kept in the dark for 15 minutes at room temperature. Two liquid-liquid extraction were then performed, each with 2 ml of diethyl ether. The organic layers were

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combined, dried under nitrogen flow and the residue was resuspended in 1 ml of acetonitrile (ACN). Quantification of PHN, CAD, HI, PUT, SPD, SPM, TRN and TY was carried out by HPLC analysis, using a Jasco HPLC apparatus (Jasco Corporation, Tokyo, Japan) and a RP Gemini C18 column (250 mm x 4.60 mm, 5 µm) (Phenomenex, Torrance, CA, USA). The mobile phase (0.8 ml min^{-1}) was composed of water and ACN and eluted with the following program: time=0 min, 65% ACN; 1 min, 65% ACN; 10 min, 80% ACN; 14 min, 80% ACN; 21 min, 100% ACN; 30 min, 100% ACN. UV detector was set at 254 nm and the injection volume was 20 µl. Standard solutions of the studied amines were used for peak identification and calibration curves calculation. LOD and LOQ were, respectively, 0.3 and 1 µg ml^{-1}. All amines calibration curves showed a good linearity in the 1 – 250 µg ml^{-1} range, as denoted by the R squared coefficients of determination (PHN, R^2=0.9997; CAD, R^2=0.9994; HI, R^2=0.9610; PUT, R^2=0.9961; SPD, R^2=0.9756; SPM, R^2=0.9732; TRN, R^2=0.9987; TY, R^2=0.9996).

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Statistical analyses

All statistical analyses were performed with R v.3.2.3 [22]. To evaluate the concordance between the qualitative screening and the HPLC quantification of BA production by the studied isolates, the McNemar exact test was used. To this end, for HPLC quantification results, the arbitrarily chosen threshold of 20 µg ml^{-1} was used, and isolates were considered positive for a specific BA production if cultured broth showed a concentration higher than the threshold value. For the concordance, only 6 of the 8 studied amines were considered, because SPD and SPM are not produced by the direct decarboxylation of an amino acid precursor, but are most commonly the results of further reactions having putrescine as a substrate [12]. Moreover, to evaluate the concordance of the two methods in detecting PUT production, only the qualitative test with ornithine addition was used as a direct comparison of the quantification of PUT production.

Results

Isolates identification

Nine out of 10 *Enterobacteriaceae* isolates were satisfactorily identified by the API 20 E kit; 8 belonged to the *Escherichia coli* species and 1 was a *Providencia rettgeri* strain. The remaining isolate was identified by 16S sequencing as *Pantoea conspicua*. Of the 34 enterococci isolates, 26 belonged to the *E. faecium* species and the remaining 8 were *E. faecalis*. For 25 out of 28 lactobacilli, identification by API 50 CH kit was successfully confirmed by species-specific PCR, and they belonged to the following species: *Lactobacillus paracasei* (n=12), *Lactobacillus*

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plantarum (n=11), *Lactobacillus fermentum* (n=1), and *Lactobacillus rhamnosus* (n=1). For the remaining 3 isolates 16S sequencing was necessary; they were 2 *Lactobacillus curvatus* and 1 *Lactobacillus coryniformis.*

Qualitative screening of BA production

In Table 2 the results of the cultivation in differential broths to detect BA production are reported. Among *Enterobacteriaceae*, all *E. coli* isolates were positive to the arginine, ornithine and tyrosine tests and thus able to produce PUT and TY. Moreover, most of them (7 out of 8) were also able to decarboxylate lysine into CAD. *P. rettgeri* was positive to the test with arginine, ornithine and lysine, while *P. conspicua* resulted negative to all tests. All *E. faecium* isolates and almost all *E. faecalis* ones resulted positive for tyrosine decarboxylation and thus TY production, and also positive to arginine and ornithine tests. Few enterococci isolates resulted positive to phenylalanine or lysine decarboxylation. Two isolates of *E. faecalis,* isolated from raw milk cheese samples, resulted positive for histidine decarboxylation and were the only isolates positive for qualitative HI production. On the other hand, lactobacilli tests were almost always negative; 3 (2 *L. curvatus* and 1 *L. paracasei*) of the 28 tested isolates were positive for tyrosine decarboxylation and only the *L. fermentum* strain resulted able to utilize arginine.

Quantitative determination of *in vitro* BA production

Results of the HPLC quantification of BA production by the tested isolates are reported in Table 3. All tested isolates were able to produce BA, although with a great degree of variability among number and quantities of BA formed. Almost all tested isolates produced TY, but while enterococci generally showed a high average production (higher than 1000 µg ml^{-1}), most *Enterobacteriaceae* and lactobacilli isolates showed very limited amounts of produced TY, with the exception of one *E. coli,* 2 *L. paracasei* and 2 *L. curvatus* isolates. In particular, both *L. curvatus* isolates had a TY production higher than 1700 µg ml^{-1}. Similarly to the qualitative screening results, lactobacilli did not show a high BA forming capacity, aside of the aforementioned TY production, and only in few cases they produced very limited quantities of CAD, PHN and TRN. Among *Enterobacteriaceae*, all *E. coli* isolates showed a notable production of CAD and PUT forming high concentrations of those amines (879 ± 943 µg ml^{-1} and 423 ± 572 µg ml^{-1}, respectively for CAD and PUT). The quantities of CAD and PUT and of PUT and TY produced, respectively, by the *P. conspicua* and *P. rettgeri* strains were instead very limited. Almost all enterococci produced significant amounts of PHN, with approximately one fourth of *E. faecium* isolates producing notable quantities of PUT and CAD, although in a lower degree compared to *Enterobacteriaceae*. Moreover, enterococci were the only tested isolates which produced HI concentrations higher than 10 µg ml^{-1}, although only in 3 cases (2 *E. faecium* and 1 *E. faecalis*) out of 34.

Concordance between the qualitative screening and the quantitative determination of BA production

Of a total of 432 paired results (6 results for each of the 72 studied isolates), 379 were in agreement (308 -/- and 71 +/+) corresponding to 87.7%. In 45 cases the qualitative screening resulted negative, while the HPLC quantification revealed an amine production. Only in 8 cases the qualitative test was positive while the quantitative determination resulted negative. Applying the McNemar test to these data confirmed that the two methods are not equivalent in detecting amine production ($p<0.000001$).

Discussion

Overall, the data from the qualitative screening and the HPLC quantification regarding *E. coli* isolates confirmed how *Enterobacteriaceae* are usually good CAD and PUT producers, both for the number of positive isolates and for the produced concentrations. These data are in agreement with those reported by other authors [11, 23]. Among *Enterobacteriaceae*, it is noteworthy that, to our knowledge, this is the first time that *P. conspicua* has been isolated from a food sample. Specifically it was isolated from a 5 month aged cheese made with raw ewes' milk. *P. conspicua* has been previously isolated only from clinical sources [24]. It has to be considered though that *P. conspicua* was only described as a new species in 2010 and was previously included in *Pantoea agglomerans* [25]. Concerning BA production, a limited production of CAD and of PUT by *P. conspicua* and *P. rettgeri,* respectively, was recorded, although these genera are considered lysine and ornithine decarboxylase and arginine dihydrolase negative [25-26]. It is true, though, that Gavini et al. [27] found 30% of *P. agglomerans* strains to be ornithine decarboxylase positive and Maifreni et al. [28] recorded CAD and PUT production by one strain of *P. agglomerans* isolated from an Italian Montasio cheese. Although not traditionally considered TY producers, production of this amine by *Enterobacteriaceae* has been previously reported [11, 29]. In our study the *E. coli* isolates and the *P. rettgeri* strain did indeed form TY, although in limited concentrations. Regarding the actual contribution of *Enterobacteriaceae* to BA accumulation in the cheeses they were isolated from, it seems that in this case their role was not a major one. Indeed, even if high levels of BA were recorded after 4 months of ripening for all cheeses, *Enterobacteriaceae* counts were always very limited with a maximum load of 1.58 ± 0.95 log CFU g^{-1} in 2 months ripened cheeses made with raw ewes' milk [13]. In fact, at the end of the ripening period, only 9 out of 36 cheeses had not neglectable (≥ 5 mg kg^{1}) CAD levels (26-55 mg kg^{1}), and this fact could reflect the limited BA formation activity of *Enterobacteriaceae* in the cheeses.

LAB, and enterococci in particular, are among the most efficient producers of tyrosine decarboxylase, the enzyme responsible for TY formation [30]. In fact, the notable production of amines recorded, and particularly TY, by

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enterococci is in agreement with previous data [7, 23, 31-32]. On the basis of chromatographic analyses and genomic data, Ladero et al. [10] have proposed that TY production can be considered a species characteristic for *Enterococcus durans, E. faecalis,* and *E. faecium*, and similarly for PUT production and *E. faecalis*. In our study all *E. faecium* and *E. faecalis* isolates produced TY and mostly in high levels. Since LAB use the agmatine deiminase pathway as the only means to produce putrescine [12], agmatine would have represented a better substrate to use, instead of ornithine or arginine, for enterococci. None the less, we surprisingly recorded a PUT production higher than 10 µg ml^{-1} in 3 out of 26 (16, 27 and 95 µg ml^{-1}) and 1 out of 8 (14 µg ml^{-1}) *E. faecium* and *E. faecalis*, respectively, and a PUT production higher than 100 µg ml^{-1} in 2 *E. faecium* (154 and 631 µg ml^{-1}) and 1 *E. faecalis* (140 µg ml^{-1}). Although BA formation can be observed in decarboxylase broths due to amino acids precursors present in the broth base itself [33], it is unlikely that the production of PUT by enterococci strains was due to agmatine presence in the broth, since agmatine is not a proteinogenic amino acid and beef extract and meat peptone were the only amino acid sources in the used broth base. Özogul and Özogul observed a good level of production of agmatine (148.15 ± 14.10 µg ml^{-1}) by *E. faecalis* in lysine decarboxylase broth, and explained it as a conversion of the arginine present in the broth, although on the contrary they did not record any agmatine production by the same strain in the arginine decarboxylase broth [33]. Currently, there is no evidence for the existence of the arginine decarboxylase in LAB [12], with the notable exception of the wine isolate *Lactobacillus hilgardii* X 1 B [34]. If agmatine was formed in the decarboxylase broth, then it could have been used by enterococci and would explain the production of PUT. As for PHN, almost all enterococci isolates (32 out of 34) in our study showed a notable production. Indeed this can be correlated to the TY production since decarboxylases often show a low amino acid specificity and can use, as a substrate, different but structurally similar free amino acids [1]. Moreover, it has been shown how in *E. faecalis* and *E. faecium* a single decarboxylase can perform both l-phenylalanine and l-tyrosine decarboxylation [35]. The production of PHN seems particularly promoted when the available tyrosine is low [30] and this fact could account for the 2 enterococci isolates in our study for which no PHN production was detected. Indeed enterococci could have had a real contribution on the BA accumulation in the cheeses they were isolated from, since PUT represented a high portion (27%) of the total recorded BA in cheeses made with raw ewes' milk, which were the only ones with average enterococci loads higher than 3 log CFU g^{-1} (4.33 ± 0.22 log CFU g^{-1}) [13].

Regarding lactobacilli, our study confirms that they are not usually good BA producers, with only a very limited number of isolates able to produce BA and even a smaller number to do so in high amounts. These data are in agreement with those previously reported [7, 23]. Even if not frequently, the production of high levels of BA, and TY in particular, by some lactobacilli strains of *Lactobacillus brevis, L. casei, L. curvatus, L. paracasei, L. plantarum, L.*

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rhamnosus and *Lactobacillus sakei* has been previously reported [7, 23, 31, 36, 37]. Strains that produce high quantities of BA, even if they have a low prevalence, can substantially contribute to amine formation if growing to high cell counts [23]. For these reasons, lactobacilli can play a major role in BA accumulation in cheese due to the high cell counts they can reach and maintain throughout the ripening period. Indeed, most of the cheeses (both raw and pasteurised milk cheeses) from which the studied microorganisms were isolated showed high levels of BA, and TY in particular, but, among the tested microbial groups, only lactobacilli counts were higher than 4.5 log CFU g^{-1} (6-7.5 log CFU g^{-1}) [13]. Moreover, even if lactobacilli are not usually described as thermoduric, their ability to survive thermal treatment and even pasteurisation has been reported in milk, which has a protective effect [38-39]. It is noteworthy that the 3 lactobacilli with the higher production of TY in our study were 1 *L. paracasei* (800 µg ml^{-1} of TY produced) and 2 *L. curvatus* (> 1700 µg ml^{-1} of TY), and all were isolated from cheeses manufactured with pasteurised milk (1 *L. curvatus* from a 2 months ripened cheese, the others from 4 months ripened cheeses) which had at the end of the ripening period an average TY content of 471 ± 271 mg kg^{1} . Indeed, *L. curvatus* comprises strains that have shown a resistance to pasteurisation [38]. Moreover, high levels of production of BA, and of TY particularly, have been previously reported for some *L. curvatus* strains, both *in vitro* and in real meat fermentations [7, 37, 40-41].

Regarding the concordance between the qualitative screening and the quantitative determination, it is known that both false positive and false negative results can occur in qualitative screening. This can either be due to the production of other basic compounds, or for the fermentative action of microorganisms and thus the acidification of the cultural medium; the latter occurrence in particular could be a major problem in the case of lactobacilli [7]. In our study the discordant results between qualitative and quantitative analysis were indeed mostly due to qualitative negative results for isolates resulted positive at the quantitative determination (45 cases out of 53 total discordant results). This can also be due to the fact that a certain amount of amine has to accumulate in the cultural medium to make the pH indicator turn. Bover-Cid and Holzapfel [7] reported 3 cases of *Lactobacillus* spp. strains that were negative to the qualitative screening although HPLC quantification showed a maximum TY production of 302 µg ml^{-1}. In this study the maximum BA production observed in the presence of a qualitative negative result were 25 µg ml^{-1}, 466 µg ml^{-1}, 631 µg ml^{-1}, and 810 µg ml^{-1}, for TRN, PHE, PUT, and TY, respectively. In our study only the qualitative tests with added ornithine were used as a comparison for the quantitative results for PUT production, although direct decarboxylation of ornithine is more distinctive of *Enterobacteriaceae*, while LAB can use other means to produce PUT [12]. On the other hand, in the case of an arginine positive qualitative test, it cannot be ruled out that the change in pH was caused by a production of agmatine which has also an alcalizing effect. Moreover, for the same reasons explained above, for enterococci the use of agmatine would represent a better qualitative screening procedure.. Overall, it seems that, even though qualitative

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screening cannot be used to reliably identify decarboxylase negative strains, it never the less represents a quick and convenient tool to discard decarboxylase positive strains.

Conclusions

Our study confirms that different microbial groups can contribute to BA formation in cheeses. *Enterobacteriaceae* and enterococci can produce notable amounts of TY, CAD and PUT. BA forming lactobacilli are more rare, but positive strains can yield high TY concentrations. Thus, BA accumulation in cheeses is not uniquely associated with undesired bacterial groups. In our study 3 lactobacilli out of 28 produced high amounts of TY, confirming that also technologically useful microorganisms, like lactobacilli, can play an important role in BA accumulation in cheeses.

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Table 1. Detailed list of the 72 isolates screened for biogenic amine production; number and source of the isolates for each bacterial group.

		Number of isolates		
	Milk	Curd	2 months ripened cheese	4 months ripened cheese
Enterobacteriaceae				
(n=10)	Pasteurised	0	2	1
	Raw	2	4	1
Enterococci				
(n=34)	Pasteurised	0	3	10
	Raw	4	7	10
Lactobacilli				
(n=28)	Pasteurised	1	3	6
	Raw	4	7	7

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Table 2. Qualitative screening of BA forming capacity of the studied isolates; number (and percentage) of positive isolates.

	Added amino acid						
	Arg	**His**	**Lys**	**Orn**	**Phe**	**Trp**	**Tyr**
Enterobacteriaceae							
Escherichia coli (n=8)	8 (100.0%)	0 (0.0%)	7 (87.5)	8 (100.0%)	2 (25.0%)	0 (0.0%)	6 (75.0%)
Pantoea conspicua (n=1)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Providencia rettgeri (n=1)	1 (100.0%)	0 (0.0%)	1 (100.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Enterococci							
Enterococcus faecium (n=26)	26 (100.0%)	0 (0.0%)	2 (7.7%)	2 (7.7%)	9 (34.6%)	0 (0.0%)	26 (100.0%)
Enterococcus faecalis (n=8)	7 (87.5%)	2 (25.0%)	0 (0.0%)	1 (12.5%)	2 (25.0%)	0 (0.0%)	7 (87.5%)
Lactobacilli							
Lactobacillus paracasei (n=12)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (8.3%)
Lactobacillus plantarum (n=11)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Lactobacillus curvatus (n=2)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (100.0%)
Lactobacillus coryniformis (n=1)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Lactobacillus fermentum (n=1)	1 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Lactobacillus rhamnosus (n=1)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Arg, arginine; His, histidine; Lys, lysine; Orn, ornithine, Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine.

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Table 3. Quantification of *in vitro* biogenic amines production by cultured isolates; μg ml^{-1} of produced amine by positive isolates (average ± standard deviation) and percentage (number) of positive isolates.

	TRN	PHN	PUT	CAD	HI	TY	SPD	SPM
Enterobacteriaceae								
Escherichia coli (n=8)	52 ± 85 62.5% (5) 5/2/1/0*	4 12.5% (1) 8/0/0/0	423 ± 572 100% (8) 0/4/2/2	879 ± 943 100% (8) 0/2/3/3	4 12.5% (1) 8/0/0/0	81 ± 185 100% (8) 0/7/1/0	– 0.0% (0) 8/0/0/0	15 12.5% (1) 7/1/0/0
Pantoea conspicua (n=1)	5 100% (1) 1/0/0/0	– 0.0% (0) 1/0/0/0	5 100% (1) 1/0/0/0	35 100% (1) 0/1/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0
Providencia rettgeri (n=1)	4 100% (1) 1/0/0/0	– 0.0% (0) 1/0/0/0	24 100% (1) 0/1/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	31 100% (1) 0/1/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0
Enterococci								
Enterococcus faecium (n=26)	7 ± 3 100% (26) 23/3/0/0	113 ± 67 92.3%(24) 3/12/11/0	185 ± 255 19.2% (5) 21/3/2/0	218 ± 143 23.1% (6) 20/2/4/0	18 ± 7 7.7% (2) 24/2/0/0	1625 ± 696 100% (26) 0/1/5/20	– 0.0% (0) 26/0/0/0	– 0.0% (0) 26/0/0/0
Enterococcus faecalis (n=8)	10 ± 4 100% (8) 4/4/0/0	399 ± 220 100% (8) 0/1/7/0	41 ± 66 50.0% (4) 6/1/1/0	3 ± 1 37.5% (3) 8/0/0/0	14 ± 16 25.0% (2) 7/1/0/0	1555 ± 952 100% (8) 0/0/3/5	– 0.0% (0) 8/0/0/0	– 0.0% (0) 8/0/0/0
Lactobacilli								
Lactobacillus paracasei (n=12)	7 ± 6 100% (12) 11/1/0/0	24 ± 28 33.3% (4) 10/2/0/0	– 0.0% (0) 12/0/0/0	30 8.3% (1) 11/1/0/0	– 0.0% (0) 12/0/0/0	171 ± 324 83.3%(10) 6/4/2/0	– 0.0% (0) 12/0/0/0	– 0.0% (0) 12/0/0/0
Lactobacillus plantarum (n=11)	6 ± 3 100% (11) 10/1/0/0	3 1 27.3% (3) 11/0/0/0	– 0.0% (0) 11/0/0/0	– 0.0% (0) 11/0/0/0	– 0.0% (0) 11/0/0/0	– 0.0% (0) 11/0/0/0	– 0.0% (0) 11/0/0/0	– 0.0% (0) 11/0/0/0
Lactobacillus curvatus (n=2)	6 ± 1 100% (2) 2/0/0/0	32 50.0% (1) 1/1/0/0	– 0.0% (0) 2/0/0/0	– 0.0% (0) 2/0/0/0	– 0.0% (0) 2/0/0/0	1862 ± 137 100% (2) 0/0/0/2	– 0.0% (0) 2/0/0/0	– 0.0% (0) 2/0/0/0
Lactobacillus coryniformis (n=1).	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0
Lactobacillus fermentum (n=1)	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	12 100% (1) 0/1/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0
Lactobacillus rhamnosus (n=1)	5 100% (1) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0	35 100% (1) 0/1/0/0	– 0.0% (0) 1/0/0/0	– 0.0% (0) 1/0/0/0

TRN, tryptamine; PHN, 2-phenylethylamine; PUT, putrescine; CAD, cadaverine; HI, histamine; TY, tyramine; SPD, spermidine; SPM, spermine; –, not detected.
*-/+/++/+++, number of strains with amine production (-), <10 μg ml^{-1}; (+), 10-100 μg ml^{-1}; (++), 100-1000 μg ml^{-1}; (+++), >1000 μg ml^{-1}.

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