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Selection of Amine-Oxidizing Dairy Lactic Acid Bacteria and Identification of the Enzyme and Gene Involved in the Decrease of Biogenic Amines

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ABSTRACT

Accumulation of biogenic amines (BAs) in cheese and other foods is a matter of public health concern. The aim of this study was to identify the enzyme activities responsible for BA degradation in lactic acid bacteria which were previously isolated from traditional Sicilian and Apulian cheeses. The selected strains would control the concentration of BAs during cheese manufacture. First, 431 isolates not showing genes encoding the decarboxylases responsible for BA formation were selected using PCR-based methods. Ninety-four out of the 431 isolates degraded BAs (2-phenylethylamine, cadaverine, histamine, putrescine, spermine, spermidine, tyramine, or tryptamine) during cultivation on chemically defined medium. As shown by random amplification of polymorphic DNA-PCR and partial sequencing of the 16S rRNA gene, 78 of the 94 strains were *Lactobacillus* species (*Lactobacillus casei*, *Lb. fermentum*, *Lb. parabuchneri*, *Lb. paracasei*, *Lb. paraplantarum*, and *Lb. rhamnosus*), *Leuconostoc* species (*Leuconostoc lactis* and *Ln. mesenteroides*), *Pediococcus pentosaceus*, *Lactococcus lactis*, *Streptococcus* species (*Streptococcus gallolyticus* and *S. thermophilus*), *Enterococcus lactis*, and *Weissella paramesenteroides*. A multicopper oxidase-hydrolyzing BA was purified from the most active strain, *Lb. paracasei* subsp. *paracasei* CB9CT. The gene encoding the multicopper oxidase was sequenced and was also detected in other amine-degrading strains of *Lb. fermentum*, *Lb. paraplantarum*, and *P. pentosaceus*. *Lb. paracasei* subsp. *paracasei* CB9CT and another strain (CACIO6CT) of the same species that was able to degrade all the BAs were singly used as adjunct starters for decreasing the concentration of histamine and tyramine in industrial Caciocavallo cheese. The results of this study disclose a feasible strategy for increasing the safety of traditional cheeses while maintaining their typical sensorial traits.

IMPORTANCE

Because high concentrations of the potentially toxic biogenic amines may be found in traditional/typical cheeses, the safety of these food items should be improved. Lactic acid bacteria selected for the ability to degrade biogenic amines may be used during cheese making to control the concentrations of biogenic amines.

Biogenic amines (BAs) are low-molecular-weight organic basic compounds formed by microbial decarboxylation of some free amino acids (FAAs) or amination and transamination of aldehydes and ketones (1, 2). On the basis of their molecular structures, they are classified as follows: (i) monoamines, including tyramine, 2-phenylethylamine, and tryptamine; (ii) diamines, such as histamine, putrescine, and cadaverine; and (iii) polyamines (spermine and spermidine) (3, 4).

Overall, BAs play a role in brain activity, regulation of body temperature and stomach pH, gastric acid secretion, the immune response, and cell growth and differentiation (5). However, the consumption of food containing large amounts of BAs may cause food intoxication (6). The negative effects of BAs on consumers' health include changes in perception, smooth muscle contractions, and/or blood pressure (5, 7). The combination of nitrites and BAs in foods results in the formation of nitrosamines, which are potential carcinogens (8).

Large amounts of BAs are usually found in fermented or spoiled foods. Recently, BAs were proposed to be food safety indicators and biomarkers for several diseases (9, 10). Among fermented foods, cheeses containing high concentration of BAs may have a serious impact on public health (11). After fish, cheese is the second most frequently reported foodstuff responsible for histamine intoxication (12, 13). A recent survey of Apulian and Sicilian cheeses revealed that about 50% of the cheeses contained at least one BA (especially histamine, followed by tyramine and putrescine) at potentially toxic concentrations (>100 mg kg^{-1}) (14).

Enterococci and heterofermentative lactobacilli are the main producers of tyramine and histamine, respectively. Other lactic acid bacteria and some Gram-negative bacteria may also be responsible for the formation of BAs in cheeses (15, 16). During

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cheese ripening, primary and secondary proteolysis releases a large amount of FAAs, some of which (e.g., Lys, Orn, His, Tyr, Trp, and Phe) are BA precursors (14, 17). Therefore, the detection of bacteria possessing enzymes that convert precursor FAAs into BAs allows the risk of food intoxication to be estimated. For this purpose, specific molecular probes and/or phenotypic assays were set up (18).

The capacity of some lactic acid bacteria to degrade BAs to aldehyde, ammonia, and hydrogen peroxide by amine oxidases (AOs) was also shown in food processing (3, 19, 20). In particular, two main classes of AOs, flavin-containing monoamine oxidases (FlavAOs) and copper-containing amine oxidases (CuAOs), have been described and are usually found in a wide range of microbes, plants, and animals (4, 21). Multicopper oxidases (MCOs) may also be responsible for BA degradation (18, 22). Previously, AOs were characterized mainly in plants and animals (4, 23, 24). Only a few studies have dealt with bacterial AOs (25).

Nowadays increasing numbers of consumers prefer traditional and typical cheeses. Most of these cheeses bear either the protected geographical indication (PGI) or the protected designation of origin (PDO). However, the manufacture of many traditional/typical cheeses relies on natural starter cultures and autochthonous microbiota. Since the members of the traditional/typical cheese microbial community are often undefined and, consequently, their corresponding metabolic activities are not known in-depth, it is not unsurprising that those cheeses could accumulate BAs (26). Therefore, the selection of autochthonous amine-negative and amine-oxidizing lactic acid bacteria (to be used as a starter or an adjunct/attenuated starter) would represent an interesting strategy for reducing the accumulation of BAs in traditional/typical cheeses while contemporarily maintaining their sensory attributes (22, 27). Once these lactic acid bacteria are characterized, they could be publicly available and, in view of implementation of the Nagoya Protocol (28), used to increase the safety of traditional/typical cheeses.

In this context, starting from hundreds of lactic acid bacteria previously isolated from 20 traditional Apulian and Sicilian cheeses (14), this study aimed to (i) select lactic acid bacteria that do not produce BAs and are able to degrade BAs, (ii) identify the enzyme and the gene involved in the degradation of BAs, and (iii) apply the selected lactic acid bacteria as a means for controlling the concentration of BAs in traditional cheese.

MATERIALS AND METHODS

Strains and culture conditions. A total of 665 isolates of presumptive lactic acid bacteria were previously isolated from Apulian traditional cheeses (Cacio, Caciocavallo Podolico Dauno, Caciocavallo Silano PDO, Cacioricotta, Canestrato Pugliese PDO, Caprino di Biccari, Caprino di Castel Fiorentino, Pecorino Foggiano, Vaccino) and Sicilian traditional cheeses (Caciocavallo Palermitano, Ragusano PDO, Caprino Girgentano, Fior di Capra, Fiore Sicano, Maiorchino, Pecorino Siciliano PDO, Piacentinu Ennese, Provola dei Nebrodi, Tuma Persa, Vastedda della Valle del Belìce PDO) (14) and were used in this study. Presumptive lactic acid bacteria were routinely propagated at 25°C (mesophilic bacteria) or 42°C (thermophilic bacteria) for 24 h in either MRS (bacilli) or M17 (cocci) broth (Oxoid Limited, Basingstoke, UK).

Selection of lactic acid bacteria not producing biogenic amine. On the basis of the findings of a previous study (14), a molecular approach was used to determine the presence/absence of genes (*tdcA*, *hdcA*, *odc*, *cadA*) responsible for BA formation (tyramine, histamine, putrescine, and cadaverine, respectively) in presumptive lactic acid bacteria. *Enterococcus faecium* DSM20477, *Lactobacillus plantarum* ATCC 14917, and *Lactobacillus paracasei* subsp. *tolerans* LMG9191 were tested for the capacity to decarboxylate Tyr, His, Orn, and Lys by detecting by high-performance liquid chromatography (HPLC) analysis the corresponding BA in the above-mentioned strains cultured (30°C, 48 h) in MRS broth supplemented with a precursor amino acid (29). *E. faecium* DSM20477 and *Lb. plantarum* ATCC 14917, able to decarboxylate Tyr and His, respectively, were used as positive controls for the *tdcA* and *hdcA* genes, respectively. *Lb. paracasei* subsp. *tolerans* LMG9191, able to decarboxylate Orn and Lys, was used as a positive control for the *odc* and *cadA* genes. DNA was extracted from bacterial cells cultured for 48 h under appropriate conditions (medium and temperature) by use of an InstaGene Matrix kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions and used as the template in the PCR. Table 1 shows for each target gene the primers (synthesized by Eurofins Genomics s.r.l., Milan, Italy) and the PCR conditions used and the size of the expected amplicons predicted by the software pDRAW32 (version 1.1.114; AcaClone Software). Amplifications were performed with a conventional thermocycler (Esco Healthcare Pte. Ltd., Singapore). PCR products were separated by electrophoresis on a 2% (wt/vol) agarose (Gibco BRL, Cergy Pontoise, France) gel and visualized by UV transillumination after staining with SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR). A GeneRuler 100-bp Plus DNA ladder (M-Medical, Milan, Italy) was used as a molecular size marker.

Selection of lactic acid bacteria degrading biogenic amines. Only isolates negative for the *tdcA*, *hdcA*, *odc*, and *cadA* genes were assayed for their capacity to degrade BAs upon cultivation in chemically defined medium (CDM), which was prepared as described by Miladinov et al. (36) with minor modifications. Modified CDM (mCDM) contained $(NH_4)_2SO_4$ (2 g liter^{-1}), Na_2HPO_4 (6 g liter^{-1}), KH_2PO_4 (3 g liter^{-1}), Na_2SO_4 (0.011 g liter^{-1}), NaCl (1 g liter^{-1}), $MgCl_2$ (0.2 g liter^{-1}), $CaCl_2$ (0.01 g liter^{-1}), and $FeCl_3 \cdot 7H_2O$ (0.0006 g liter^{-1}) (Merck, Milan, Italy). After sterilization, the following were added: filter-sterilized glucose (5 g liter^{-1}; Oxoid) and biotin (0.0001 g liter^{-1}); folic acid, riboflavin, nicotinic acid, thiamine, and pantothenic acid (0.001 g liter^{-1} each); pyridoxal (0.002 g liter^{-1}); and sodium acetate (2 g liter^{-1}) (Carlo Erba Reagenti, Milan, Italy). mCDM was supplemented with one of the following BAs: tyramine (2.5 mM); histamine (1 mM); or putrescine, cadaverine, tryptamine, 2-phenylethylamine, spermine, or spermidine (0.54 mM each) (Sigma-Aldrich, Milan, Italy) (25, 27). mCDM without BA was used as the negative control for bacterial growth. Isolates were precultured twice in MRS or M17 broth. Cells were centrifuged at 10,000 × *g* for 5 min at 4°C, washed twice in Ringer's solution, and inoculated (initial cell density, ca. 7 log CFU g^{-1}) in mCDM broth. After incubation (at either 25 or 42°C for 48 to 72 h), bacterial growth was measured by reading the absorbance at a wavelength of 600 nm through a 6400 spectrophotometer (Jenway Ltd., Felsted Dunmow, UK). Bacterial growth was confirmed through pour plating (in MRS or M17 agar medium, depending on the isolation medium) of the mCDM liquid cultures at the beginning and end of incubation. In addition, the capacity of 19 isolates to degrade BAs was assessed by HPLC (25) at ISVEA s.r.l. (Poggibonsi, Siena, Italy), using ISVEA protocols. The percentage of BA that was degraded was calculated on the basis of the peak areas corresponding to tyramine, histamine, putrescine, cadaverine, tryptamine, 2-phenylethylamine, spermine, and spermidine soon after inoculation of mCDM and after incubation (27).

Genotypic characterization and identification of amine-degrading bacteria. Decarboxylase-negative, amine-degrading lactic acid bacteria were typed by random amplification of polymorphic DNA (RAPD)-PCR using primer M13 (37). PCR was performed under the conditions described by Zapparoli et al. (38). The PCR products were separated by electrophoresis and visualized as described above. RAPD-PCR profiles were analyzed by the pattern analysis software package GelCompar II (version 6.5; Applied Maths, Sin Marten Latem, Belgium). Calculation of the similarities of the band profiles was based on the Pearson product moment correlation coefficient. Dendrograms were obtained by

TABLE 1 Primers having as targets genes coding for enzymes involved in the formation and degradation of BAs and PCR conditions adopted in this work

Enzyme	Primer name	Target gene	Sequence (5′-3′)	Reference or source	Expected amplicon size (bp)	Amplification program
Tyrosine decarboxylase	TDC1	*tdcA*	AACTATCGTATGGATATCAACG	30	700	94°C for 2 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; 72°C for 5 min
	TDC2		TAGTCAACCATATTGAAATCTGG			
Histidine decarboxylase	Hdc1	*hdcA*	TTGACCGTATCTCAGTGAGTCCAT	31	174	94°C for 2 min; 35 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 30 s; 72°C for 5 min
	Hdc2		ACGGTCATACGAAACAATACCATC			
Ornithine decarboxylase	ODC1	*odc*	NCAYAARCAACAAGYNGG	32	830	94°C for 2 min; 35 cycles of 94°C for 30 s, 54°C for 45 s, and 72°C for 1 min 15 s; 72°C for 5 min
	ODC2		GRTANGGNTNNGCACCTTC			
Lysine decarboxylase	CadAf	*cadA*	GCTGGGTTCACTGCTGGA	33	220	94°C for 2 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s; 72°C for 5 min
	CadAr		TGGCGTAATGTAGCTCATCA			
Lysine decarboxylase	Cad2F[a]	*ldc*	CAYRTNCCNGGNCAYAA	34	1,185	94°C for 2 min; 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min 30 s; 72°C for 5 min
	Cad2R[a]		GGDATNCCNGGNGGRTA			
Agmatine deiminase	AgmSq1[a]	*agu*	CAAGATTTDTTCTGGGCHTTYTTCTC	35	700	94°C for 2 min; 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; 72°C for 5 min
	AgmSq2[a]		TTGGHCCACARTCACGAACCCT			
Agmatine deiminase	AgD1[a]	*agdA*	CAYGTNGAYGGHSAAGG	32	600	94°C for 2 min; 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; 72°C for 5 min
	AgD2[a]		TGTTGNGTRATRCAGTGAAT			
Multicopper oxidase	BC1f[a]	*sufI*	CGTTTGAAGGGGGAAATCAC	This study	1,570	94°C for 2 min; 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min 40 s; 72°C for 5 min
	BC1r[a]		TATATTAGTATTGTGGGACG			
Multicopper oxidase	BC2f[a]	*sufI*	CTTTGATGAGCCAGCGTTTG	This study	1,436	94°C for 2 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min 30 s; 72°C for 5 min
	BC2r[a]		GCATACCGCCCATCCAAATC			
Multicopper oxidase	BC3f[a]	*sufI*	GAGGCTGGGGAAACGCAACT	This study	332	94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; 72°C for 5 min
	BC3r[a]		CTTGGCAATGGCAGGCTGGC			
Multicopper oxidase	BC4f[a]	*sufI*	CAGGGGATGGACGAAGGTGT	This study	329	94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; 72°C for 5 min
	BC4r[a]		TCTTGCTTTGGCTTGGCTGG			

[a] Primers used only with DNA extracted from amine-degrading strains of lactic acid bacteria.

means of the unweighted pair group method using an arithmetic average (UPGMA) clustering algorithm.

Genotypic identification of the lactic acid bacteria was carried out by partial sequencing of the 16S rRNA gene, as described by Weisburg et al. (39) and Corsetti et al. (40). Primers casei (forward), para (forward), and Y2 (reverse) (Eurofins Genomics) were used to identify species in the *Lactobacillus casei* group (41). PCR products were purified by use of a QIAquick purification kit (Qiagen S.p.a., Milan, Italy) and sequenced by Eurofins Genomics. Comparative searches against the sequences available in the GenBank/EMBL/DDBJ databases were performed using the Basic Local Alignment Search Tool (BLAST) program (42).

Use of additional primers targeting other genes involved in the formation of putrescine and cadaverine. Since putrescine may be produced not only from ornithine but also from agmatine (43), the amine-degrading strains identified were further tested using two pairs of primers (AgmSq1/AgmSq2 and AgD1/AgD2; Table 1) targeting genes (*agu* and *agdA*, respectively) involved in the agmatine deiminase pathway. In addition, these strains were tested using an additional pair of primers (Cad2F/Cad2R; Table 1) targeting the Lys decarboxylase gene. *Lactobacillus brevis* ATCC 367 and *E. faecium* ATCC BAA-472, capable of producing putrescine and cadaverine from agmatine and Lys (29), respectively, were used as positive controls.

Identification of amine-degrading enzyme. Among the amine-degrading lactic acid bacteria, the most active strain, *Lb. paracasei* subsp. *paracasei* CB9CT, was selected for the identification of the amine-degrading enzyme. Cytoplasmic proteins were extracted from cells of strain CB9CT, according to the method described by Gobbetti et al. (44) with some modifications. In detail, bacterial cells cultured (24 h) in 1 liter of MRS broth were collected by centrifugation (15,300 × *g*, 10 min, 4°C) and washed twice with 50 mM Tris-HCl, pH 7.5, buffer. Cells were further suspended in the same buffer containing sucrose (25%, wt/vol; Sigma-Aldrich). The cell suspension was incubated (37°C, 30 min) with stirring (120 rpm), and finally, lysozyme (1 g liter^{-1}; Sigma-Aldrich) was added. After cell wall lysis (37°C for 1 h with stirring), the suspension was centri-

fuged (37°C, 30 min, 12,000 × *g*). The pellet was suspended in 2 ml of 50 mM Tris-HCl, pH 7.5, buffer. After incubation (30°C, 30 min), the suspension was kept in an ice bath and treated ultrasonically with a Vibra-Cell sonicator (Sonic and Materials Inc., Danbury, CT, USA) equipped with a microtip setting (sonic power, 375 W; output control, 5) for 15 min (3 cycles, 5 min/cycle). Subsequently, the suspension was centrifuged (10,170 × *g*, 4°C, 10 min), and 6 ml of cold acetone was added to the supernatant, which putatively contained cytoplasmic proteins. After overnight incubation at −20°C, the precipitated proteins were collected by centrifugation (19,940 × *g*, 4°C, 10 min), washed with acetone, and air dried for 10 min.

Proteins were resuspended in 50 mM sodium acetate, pH 5.6, buffer and assayed for BA oxidase activity by the colorimetric assay reported by Foster et al. (45), with minor modifications. In detail, 40 μl of partially purified enzyme, 400 μl of 4-aminoantipyrine (1.5 mM; Sigma-Aldrich), and 2,4,6-tribromo-3-hydroxybenzoic acid (1 mM; Sigma-Aldrich) in 200 mM potassium phosphate, pH 7.6, buffer were mixed. Eighty microliters of histamine (100 mM) and 280 μl of horseradish peroxidase (0.8 U ml^{-1} of reaction mixture) (Sigma-Aldrich) were added to initiate the enzyme reaction. The reaction mixture was incubated at 30°C for 45 min and subjected to measurement of the absorbance at a wavelength of 510 nm. A positive control was represented by a reaction mixture wherein partially purified enzyme was replaced by commercial diamine oxidase from porcine kidney (Sigma-Aldrich). A negative control, consisting of a reaction mixture wherein histamine was replaced by a solution of potassium phosphate buffer, was also included.

The protein extract was applied to a DEAE-cellulose anion exchange column (55 by 1.6 cm; GE Healthcare, Uppsala, Sweden). Proteins were eluted with a linear NaCl gradient (0 to 0.3 M) in 50 mM sodium acetate, pH 5.6, buffer at a flow rate of 42 ml h^{-1}. The fractions with the highest oxidase activity were pooled, dialyzed against 5 mM sodium acetate, pH 5.6, buffer, concentrated 10-fold by freeze-drying, and subjected to gel filtration on a fast protein liquid chromatography (FPLC) Superose 12HR 10/30 column (GE Healthcare). Elution with 50 mM sodium acetate, pH 5.6, buffer containing 0.15 M NaCl was at a flow rate of 18 ml h^{-1}. Active fractions were pooled, dialyzed against 50 mM sodium acetate, pH 5.6, buffer, and, finally, applied to an FPLC Mono-Q HR 5/5 column (GE Healthcare), and elution with a linear gradient of NaCl from 0 to 0.5 mM in the same buffer was performed at a flow rate of 24 ml h^{-1}. The protein concentration during the purification steps was determined as described by Bradford (46).

The active fraction was digested by trypsin (trypsin from bovine pancreas suitable for protein sequencing; Sigma-Aldrich), and the resulting peptides were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an Q-Exactive Orbitrap mass spectrometer at Bioproximity, LLC (Chantilly, VA, USA). Protein identification was carried out by using the Amazon web services-based Cluster Compute instances and the Proteome Cluster interface. The protein sequence was analyzed by use of a search of the sequences in the GenBank/EMBL/DDBJ (RefSeq) and UniProtKB (www.uniprot.org/help/uniprotkb) databases with the BLAST program and aligned to the sequences of similar proteins from lactic acid bacteria by use of the ClustalW (version 1.83) program (47). The alignment output files were formatted using BioEdit (version 7.0.9) software (http://www.mbio.ncsu.edu/BioEdit/). Protein domains were located using the Pfam database (48) (http://pfam.sanger.ac.uk), the Simple Modular Architecture Research Tool (SMART) database (http://smart.embl-heidelberg.de) (49), and the Conserved Domain Database (CDD; http://www.ncbi.nlm.nih.gov/cdd/).

Detection of genes encoding amine-degrading enzyme. Four pairs of primers (Eurofins Genomics) were designed on the basis of the amino acid sequence of the amine-degrading enzyme (Table 1). Primers mapping to internal regions (BC2 to BC4) were used in nested amplifications, in order to verify the nature of the identified gene and to obtain its complete sequence. To detect the presence of the identified amine-degrading enzyme of *Lb. paracasei* subsp. *paracasei* CB9CT in the other amine-degrading strains, genomic DNA from selected strains (one for each species) was used as the template for amplification. PCR products were separated, visualized, and identified by sequencing as described above.

Additionally, degenerated primers (Eurofins Genomics) targeting AO genes from *Firmicutes* and other bacteria were designed and tested in different combinations (see the supplemental material).

Experimental cheese manufacture and analysis of biogenic amines contained in cheese. Caciocavallo-type cheese was manufactured at the industrial plant Ignalat (in a vat of about 200 liters), located in Noci (Bari, Italy). Pasteurized cow's milk was held at 37°C and used to manufacture six types of experimental cheeses using different cultures combinations: (i) commercial *Streptococcus thermophilus* (Sacco, Cadorago, Como, Italy) and the histamine producer *Lactobacillus fermentum* CCS12BT; (ii) *S. thermophilus* and *Lb. paracasei* subsp. *paracasei* CB9CT (capable of growing on mCDM to which tyramine, histamine, tryptamine, or spermine was added); (iii) *S. thermophilus*, *Lb. paracasei* subsp. *paracasei* CB9CT, and *Lb. fermentum* CCS12BT; (iv) *S. thermophilus* and *Lb. paracasei* subsp. *paracasei* CACIO6CT (capable of growing on mCDM to which any of the BAs was added); (v) *S. thermophilus*, *Lb. paracasei* subsp. *paracasei* CACIO6CT, and *Lb. fermentum* CCS12BT; and (vi) *S. thermophilus* only (control cheese). All strains were inoculated at ca. 7 log CFU ml^{-1} of milk. Coagulation took place 30 min after addition of liquid calf rennet (0.0001%, vol/vol). The coagulum was first cut coarsely by hand, held under whey at 37°C for 2 h, and then reduced to particles of 1.5 to 2 cm. When the curd reached a pH of 5.25 (after ca. 5 h at room temperature), it was stretched (at ca. 65°C for 5 min) in hot (80°C) water. The cheeses were salted in brine (30% [wt/vol] NaCl) for 12 h. Ripening was at ca. 10°C and a relative humidity of 83% for 60 days. The weight of the cheese was approximately 1.5 kg.

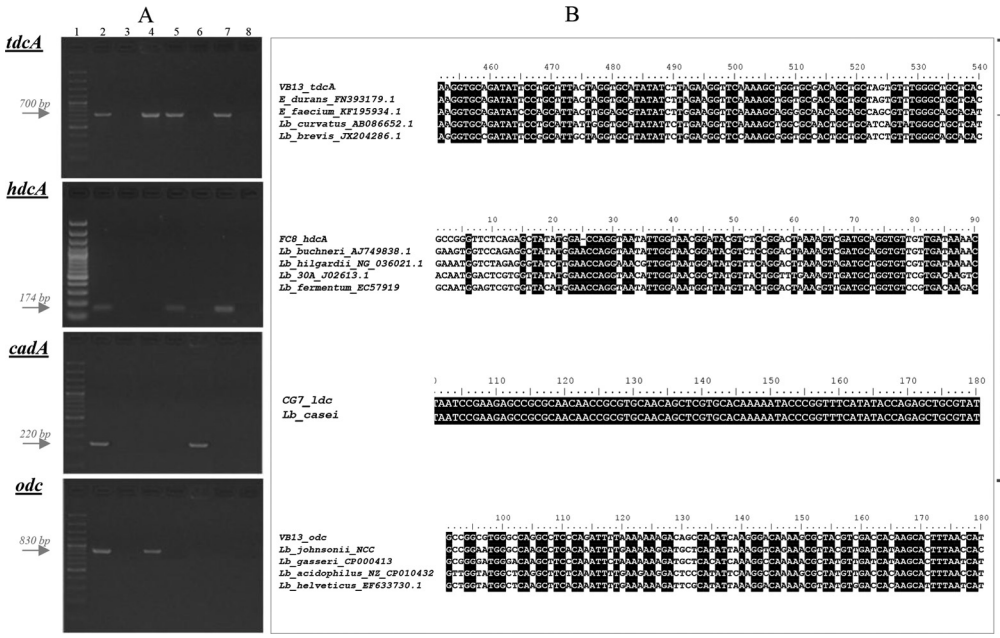
The presumptive thermophilic or mesophilic lactic acid bacteria in the experimental cheeses were enumerated 1 day after manufacture and at 30 and 60 days of ripening, as described by Di Cagno et al. (50). The ripened cheeses were analyzed for the content of protein (by the macro-Kjeldahl method) (51), fat (by the Gerber method) (52), moisture (by oven drying at 102°C) (53), and salt (54). The pH was determined by direct insertion of a FoodTrode (Hamilton, Bonaduz, Switzerland) electrode. The concentrations of BAs in the ripened cheeses were determined by HPLC at ISVEA s.r.l. (Poggibonsi, Siena, Italy), using ISVEA protocols. Sensory analysis of the experimental cheeses (control and cheese to which CB9CT was added) was carried out by using the triangle test (the ISO 4120:2004 standard protocol). In detail, 42 assessors were recruited, and the results were interpreted according to the statistical table, Table A.1, annexed to the above-cited standard protocol (ISO 4120:2004), by choosing an α value for the level of risk of 0.05 (50).

Statistical analyses. Data were subjected to one-way analysis of variance, and pairwise comparison of treatment means was achieved by Tukey's procedure and by use of a P value of <0.05, using the statistical software Statistica (version 7.0 for Windows).

Accession number(s). The 16S rRNA partial gene sequences of the bacteria studied have been deposited in the GenBank database under accession numbers KC545945, KC545887, KC545888, KC545892, KC545908, KC545910, KC545924, KC545927, KC545931, KF147888, KC545935, KC545941, JN696705, KF060258, HQ721247, and KU315055 to KU315117 (see also Table S4 in the supplemental material).

RESULTS

Selection of lactic acid bacteria not producing biogenic amines. Preliminarily, 665 isolates from a previous study (14) were screened by PCR, using primers targeting genes encoding enzymes decarboxylating amino acid precursors of BAs (see Table S2 in the supplemental material). About 65% of the isolates did not show amplicons. The remaining aliquots produced amplicons of the expected sizes. Sequencing confirmed the high degree of homology of the amplicons to genes (*tdcA*, *hdcA*, and *odc*) encoding decarboxylases (tyrosine decarboxylase [EC 4.1.1.25], histidine



Isolates from cheese	N. of isolates harbouring at least one gene encoding enzymes involved in the formation of BA	N. of isolates harbouring no gene encoding enzymes involved in the formation of BA
Cacio	21	19 (47.5)
Caciocavallo Palermitano	17	15 (46.8)
Caciocavallo Podolico Dauno	3	27 (92.5)
Caciocavallo Ragusano	3	12 (80.0)
Caciocavallo Silano PDO	24	22 (47.8)
Cacioricotta	10	30 (75.0)
Canestrato Pugliese PDO	12	17 (58.6)
Caprino di Biccari	23	28 (54.9)
Caprino di Castel Fiorentino	7	28 (80.0)
Caprino girgentano	4	26 (81.2)
Fior di capra	5	17 (77.3)
Fiore sicano	8	34 (80.9)
Maiorchino	14	8 (36.4)
Pecorino Foggiano	18	26 (59.1)
Pecorino Siciliano PDO	13	10 (43.5)
Piacentinu Ennese	8	12 (60.0)
Provola dei Nebrodi	5	14 (73.7)
Tuma persa	8	23 (74.2)
Vaccino	14	33 (70.2)
Vastedda della Valle del Belice	5	31 (86.1)

FIG 1 (A) PCR-based detection of genes (*tdcA*, *hdcA*, *cadA*, *odc*) encoding enzymes involved in the formation of biogenic amines (tyramine, histamine, cadaverine, putrescine) in lactic acid bacteria. Lanes: 1, molecular weight marker (100 to 3,000 bp); 2, PCR positive controls (*Enterococcus faecium* DSM20477 for *tdcA*, *Lactobacillus plantarum* ATCC 14917 for *hdcA*, *Lb. paracasei* subsp. *tolerans* LMG9191 for *odc* and *cadA* genes); 3, PCR negative controls; 4, VB13 (presumptive lactic acid bacterium isolated from Vastedda della Valle del Belice cheese); 5, FC8 (presumptive lactic acid bacterium isolated from Fior di Capra cheese); 6, CG7 (presumptive lactic acid bacterium isolated from Caprino Girgentano cheese); 7, CP3 (presumptive lactic acid bacterium isolated from Caciocavallo Palermitano cheese); 8, TP5 (presumptive lactic acid bacterium isolated from Tuma Persa cheese). (B) Multiple-sequence alignments of the sequences obtained by PCR targeting genes encoding enzymes involved in the formation of biogenic amines (entries) and the sequences saved in the GenBank database. (C) Cheeses and the number of bacterial isolates from Apulian or Sicilian cheeses harboring at least one (or no) gene coding for enzymes involved in the formation of histamine, cadaverine, putrescine, and tyramine. For each cheese, the percentage of isolates not producing biogenic amines among the total number of isolates is shown in parentheses.

decarboxylase [EC 4.1.1.22], and ornithine decarboxylase [EC 4.1.1.17], respectively) from lactic acid bacteria (Fig. 1A). The only exception was found for the amplicons targeting the *cadA* gene (encoding lysine decarboxylase [EC 4.1.1.18]). The sequence obtained was similar to the sequence of the region from positions 1196937 to 1197175 of the *Lb. casei* W56 genome (HE970764), which codes for a putative uncharacterized protein (Fig. 1B).

By considering the total number of isolates from a given cheese, the proportion of isolates not producing a BA varied from ca. 36% (Maiorchino cheese) to 92.5% (Caciocavallo Podolico Dauno cheese) (Fig. 1C). All (n = 431) presumptive lactic acid bacteria not producing a BA were selected and used for further characterization.

Selection of lactic acid bacteria degrading biogenic amines. The capacity of selected presumptive lactic acid bacteria to degrade BAs was assayed through cultivation on mCDM supplemented with tyramine, histamine, putrescine, cadaverine, tryptamine, 2-phenylethylamine, spermine, or spermidine. As assessed through indirect methods (spectrophotometry) and direct methods (plate counting), no isolates from Caciocavallo Podolico Dauno, Caciocavallo Silano PDO, Cacioricotta, Canestrato Pugliese PDO, Caprino di Castel Fiorentino, and Vaccino cheeses degraded BAs. On the contrary, all the isolates from Provola dei Nebrodi cheese not producing BAs were able to degrade one or more BAs (see Table S3 in the supplemental material). Ninety-four out of the 431 isolates not producing BAs degraded at least one BA. Histamine, tyramine, putrescine, and cadaverine were the most commonly used BAs. In detail, the proportion of presumptive lactic acid bacteria able to degrade histamine ranged from 40% (e.g., Tuma Persa cheese) to 100% (e.g., Fior di Capra cheese) (see Table S3). The proportion of isolates able to degrade tyramine, putrescine, or cadaverine was found to be in the same range, except for isolates from Caprino di Biccari cheese (20% degraded tyramine, no isolate degraded putrescine or cadaverine) and Piacentinu Ennese cheese (no activity toward cadaverine). The amine-degrading capacity of 19 out of 94 strains was confirmed by HPLC. The rate of degradation ranged from ca. 21 to 45% (data not shown).

Genotypic characterization and identification of amine-degrading lactic acid bacteria. Seventy-eight of the 94 presumptive amine-degrading lactic acid bacterial isolates not producing BAs showed different RAPD profiles (data not shown). Consequently, all these isolates were subjected to identification by partial sequencing of the 16S rRNA gene. The gene sequences were deposited in GenBank and may be found under the accession numbers indicated above. Numerically, the majority of the strains belonged to the *Lb. paracasei* (ca. 47%) and *Leuconostoc mesenteroides* (ca. 16%) species. Other strains identified were *Enterococcus lactis*, *Lb. brevis*, *Lb. casei*, *Lb. fermentum*, *Lactobacillus parabuchneri*, *Lactobacillus paraplantarum*, *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Leuconostoc lactis*, *Pediococcus pentosaceus*, *Streptococcus gallolyticus*, *S. thermophilus*, and *Weissella paramesenteroides*. Because putrescine may also be derived from agmatine (32), two pairs of additional primers (AgmSq1/AgmSq2 and AgD1/AgD2; Table 1)

TABLE 2 Substrate specificity of enzymatic activities and presence/absence of the gene encoding multicopper oxidase in amine-degrading strains of lactic acid bacteria isolated from Apulian or Sicilian cheese

Strain	Growth on[a]:								Recovery of a PCR product of the expected size
	Tyr	His	Put	Cad	Phe	Try	Sp	Spd	
Enterococcus lactis FC5	+	+	−	+	−	+	−	−	−
Lactobacillus brevis PE1	+	+	+	−	+	+	+	+	−
Lactobacillus casei PN5	+	+	−	−	−	−	−	−	−
Lactobacillus fermentum PE2	−	−	−	−	−	−	+	−	+
Lactobacillus parabuchneri TP3	−	−	−	−	+	+	−	−	−
Lactobacillus paracasei subsp. *paracasei* CB9CT	+	+	−	−	−	+	+	−	+
Lactobacillus paraplantarum R5	−	+	−	+	−	−	+	+	+
Lactobacillus rhamnosus TP5	−	−	−	−	−	−	+	+	−
Lactococcus lactis VB2	−	−	+	−	+	+	+	+	−
Leuconostoc lactis VB8	+	+	+	+	−	−	−	−	−
Leuconostoc mesenteroides subsp. *mesenteroides* PF13CM	+	+	+	+	+	+	+	+	−
Pediococcus pentosaceus M1	+	+	+	+	−	−	−	−	+
Streptococcus gallolyticus subsp. *macedonicus* R1	+	+	+	+	−	−	+	+	−
Streptococcus thermophilus PF3CT	−	+	−	+	−	−	−	−	−
Weissella paramesenteroides CP2	+	+	+	+	+	+	+	+	−

[a] Abbreviations: Tyr, modified chemically defined medium (mCDM) plus tyramine; His, mCDM plus histamine; Put, mCDM plus putrescine; Cad, mCDM plus cadaverine; Phe, mCDM plus phenylethylamine; Try, mCDM plus tryptamine; Sp, mCDM plus spermine; Spd, mCDM plus spermidine.

were used in PCR experiments in which DNA extracted from the amine-degrading strains was used as the template. Additional primers (Cad2F/Cad2R; Table 1) targeting the *ldc* gene, encoding the Lys decarboxylase of Gram-positive bacteria, were used to confirm that the amine-degrading strains did not harbor the *ldc* gene. Molecular assays based on the results of PCR with those additional primers confirmed that none of the amine-degrading strains was able to produce putrescine and cadaverine from agmatine and lysine, respectively (data not shown). For Pecorino Foggiano, Caciocavallo Ragusano PDO, Caprino Girgentano, Caprino di Biccari, and Cacio cheeses, strains mostly belonged to one of two species (*Ln. mesenteroides* or *Lb. paracasei*). The other cheeses showed a greater microbial species diversity.

Seventeen strains that were isolated from seven different cheeses and that belonged to different genera of lactic acid bacteria were able to grow on mCDM supplemented with any of the BAs. However, substrate specificity was generally observed and strongly depended on the strain. For instance, two strains (VB5 and VB12) of *Ln. mesenteroides* were not able to degrade histamine and cadaverine, which is different from the findings for other strains of the same species (e.g., PN7, VB6, and VB7). Similarly, two strains (TP4 and FC6) of *P. pentosaceus* were able to use 2-phenylethylamine, whereas strains PSL55 and M1, belonging to the same species, were not. *Lb. paracasei* subsp. *paracasei* CB9CT showed the highest amine-degrading activity when cultured in mCDM containing histamine (data not shown). In addition, this strain was able to degrade tyramine, tryptamine, and spermine. Therefore, it was selected for use in further studies.

Purification of *Lb. paracasei* subsp. *paracasei* CB9CT multicopper oxidase. A multicopper oxidase family protein (otherwise annotated as a blue copper oxidase) of 509 amino acid residues from *Lb. paracasei* subsp. *paracasei* CB9CT was purified and identified by LC-MS/MS (Fig. 2). Sequence analysis revealed three characteristic cupredoxin domain repeats (CuRO_CueO_FtsP, consisting of the cupredoxin domain of the multicopper oxidase CueO, the cell division protein FtsP, and similar proteins) that include one mononuclear and one trinuclear copper center. Overall, the protein was similar to a number of proteins of the same family identified in bacteria phylogenetically related to *Lb. paracasei*. The highest similarity (≥99.6%) was found with the multicopper oxidases of *Lb. paracasei* (WP_016384138.1), *Lb. casei* (WP_003585581.1), and *Lb. plantarum* (WP_046041228) (Fig. 2).

Detection of genes encoding amine-degrading enzyme. A genomic fragment of 1,571 bp which corresponded to the full coding sequence of the *Lb. paracasei* subsp. *paracasei* CB9CT multicopper oxidase gene was obtained using primer pair BC1f/BC1r (GenBank accession number KU962939). The other pairs of primers (BC2f/BC2r, BC3f/BC3r, and BC4f/BC4r) gave positive amplification results as well. The gene sequence shared 99% and 98% identity with the sequences of *Lb. paracasei* L9 (GenBank accession number CP012148.1) and *Lb. casei* W56 (GenBank accession number HE970764.1), respectively. The same amplification product was found for other strains belonging to different species, including *Ln. mesenteroides*, *Lb. paraplantarum*, *Lb. fermentum*, and *P. pentosaceus* (Table 2). Strikingly, no amplification occurred for other strains of *E. lactis*, *Lb. brevis*, *Lb. casei*, *Lb. parabuchneri*, *Lb. rhamnosus*, *Lc. lactis*, *Ln. lactis*, *Ln. mesenteroides*, *S. gallolyticus*, *S. thermophilus*, and *W. paramesenteroides* which were capable of growing on mCDM supplemented with BAs. No amplification product was observed when genomic DNA extracted from the isolate *Lb. paracasei* CB3BT, which was not able to degrade BAs (negative control), was used as the template.

Biogenic amine degradation during cheese ripening. *Lb. paracasei* subsp. *paracasei* CB9CT, which had the highest level of histamine-degrading activity, and *Lb. paracasei* subsp. *paracasei* CACIO6CT, which degraded all the BAs, were singly used as adjunct starters for making experimental Caciocavallo cheese. Each strain was inoculated together with the commercial starter (*S. thermophilus*) alone (CB9CT/CACIO6CT cheese) or with this primary starter in combination with the histamine producer *Lb. fermentum* CCS12BT (CCS12BT + CB9CT/CACIO6CT cheese). Furthermore, a control cheese started only with *S. thermophilus* and a cheese started with *S. thermophilus* and *Lb. fermentum* CCS12BT (CCS12BT cheese) were also manufactured. The latter

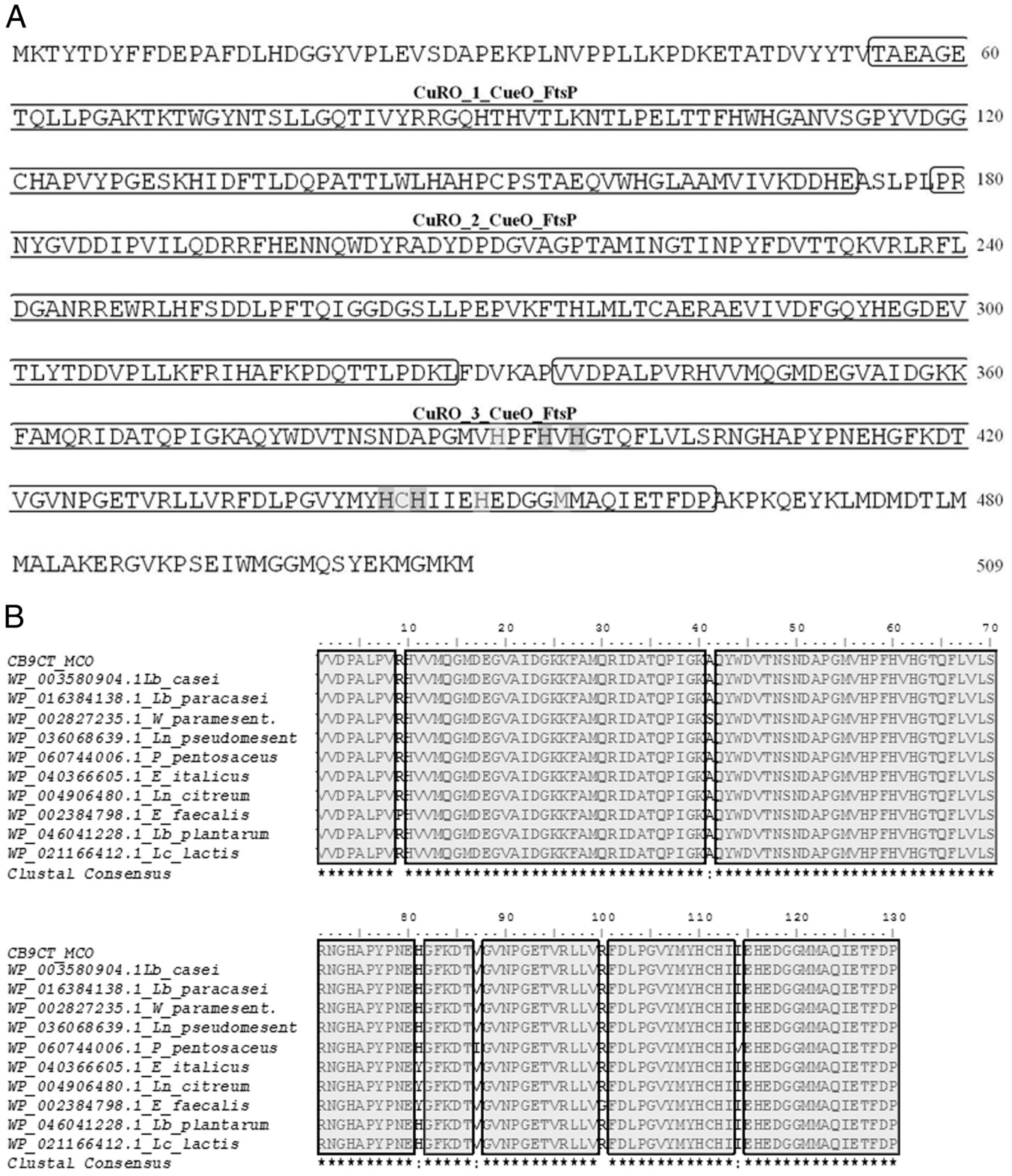


FIG 2 (A) Multicopper oxidase enzyme purified from *Lactobacillus paracasei* subsp. *paracasei* CB9CT. Type 1 (T1) and trinuclear copper binding sites are highlighted in light gray and dark gray, respectively. (B) Multiple-sequence comparison of the CuRO_3_CueO_Ftsp domains of some lactic acid bacterial species. Identical residues are outlined and indicated by asterisks *W_paramesent.*, *Weissella paramesenteroides*; *Ln_pseudomesent*, *Leuconostoc pseudomesenteroides*.

TABLE 3 Cell densities of presumptive mesophilic and thermophilic lactobacilli, mesophilic lactococci, and thermophilic streptococci at different times after ripening in experimental Caciocavallo cheeses manufactured with commercial bacteria alone or in combination with autochthonous adjunct starter bacteria[a]

	Cell density[b] (no. of log CFU g^{-1})											
	Mesophilic lactobacilli			Thermophilic lactobacilli			Mesophilic lactococci			Thermophilic streptococci		
Cheese	T1	T30	T60	T1	T30	T60	T1	T30	T60	T1	T30	T60
Control	5.0C	5.8D	7.3C	6.4A	7.3AB	8.0A	7.0A	4.8BC	1.8A	8.5A	7.0A	4.7A
CCS12BT	5.6B	6.5BC	8.1AB	5.9B	7.4AB	8.2A	6.8B	5.2A	1.6AB	8.3A	7.0A	4.5A
CB9CT	5.5B	6.3C	8.0B	6.0B	7.5A	8.3A	6.7B	4.6C	1.5B	8.3A	6.9A	4.8A
CCS12BT + CB9BT	6.1A	6.8AB	8.2A	6.2AB	7.4AB	8.2A	7.1A	5.0AB	1.6AB	8.2A	6.8A	4.6A
CACIO6CT	5.7B	6.5BC	8.2A	6.2AB	7.1B	8.0A	7.2A	5.2A	1.9A	8.5A	7.1A	4.8A
CCS12BT + CACIO6CT	6.3A	7.0A	8.4A	6.3AB	7.5A	8.1A	6.9AB	5.0AB	1.7A	8.4A	6.8A	4.6A

[a] The bacteria were commercial *Streptococcus thermophilus* (control), *S. thermophilus* and autochthonous *Lactobacillus fermentum* CCS12BT (CCS12BT), *S. thermophilus* and autochthonous *Lactobacillus paracasei* subsp. *paracasei* CB9CT (CB9CT), *S. thermophilus*, *Lb. fermentum* CCS12BT, and *Lb. paracasei* subsp. *paracasei* CB9CT (CCS12BT + CB9CT), *S. thermophilus* and autochthonous *Lb. paracasei* subsp. *paracasei* CACIO6CT (CACIO6CT), or *S. thermophilus*, *Lb. fermentum* CCS12BT, and *Lb. paracasei* subsp. *paracasei* CACIO6CT (CCS12BT + CACIO6CT). T1, T30, and T60, 1, 30, and 60 days of ripening, respectively.
[b] Values in the same column with different letters are significantly different ($P < 0.05$).

cheese was manufactured in order to ensure the production of BAs. One day after manufacturing of the cheese, as well as after 30 and 60 days of ripening, the cell density of presumptive mesophilic lactobacilli in the cheeses produced with adjunct starters was higher ($P < 0.05$) than that of the control (Table 3). The amounts of mesophilic and thermophilic lactobacilli increased during ripening, and they thus represented the dominant groups of lactic acid bacteria. On the contrary, the amounts of presumptive streptococci and, especially, lactococci decreased during ripening. As expected, the highest concentration of tyramine and, especially, the highest concentration of histamine were found in the CCS12BT cheese (Fig. 3). All the cheeses manufactured with an amine-degrading strain (CB9CT or CACIO6CT) showed concentrations of histamine and tyramine lower ($P < 0.05$) than those seen in the control and CCS12BT cheeses. The lowest concentrations of histamine and tyramine were found in CB9CT cheese. No significant differences ($P > 0.05$) in the concentrations of cadaverine and putrescine in the experimental Caciocavallo cheeses were found. Other BAs were not detected in any cheese. The gross composition and pH were not significantly ($P > 0.05$) different among these cheeses (see Table S5 in the supplemental material).

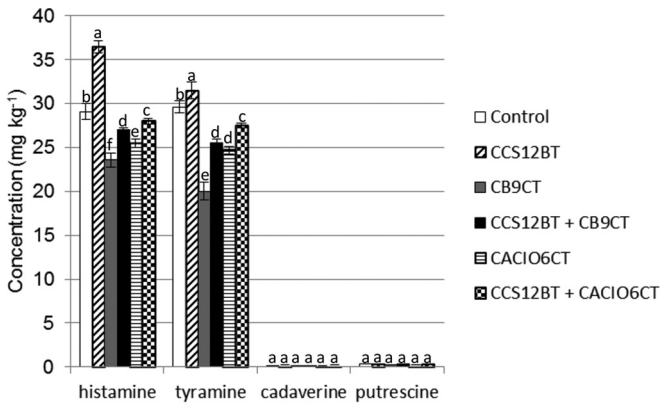


FIG 3 Concentrations of histamine, tyramine, cadaverine, and putrescine in the experimental Caciocavallo cheeses ripened for 60 days. Control, Caciocavallo cheese inoculated with commercial *Streptococcus thermophilus*; CCS12BT, Caciocavallo cheese inoculated with *S. thermophilus* and *Lactobacillus fermentum* CCS12BT; CB9CT, Caciocavallo cheese inoculated with *S. thermophilus* and *Lactobacillus paracasei* subsp. *paracasei* CB9CT; CCS12BT + CB9CT, Caciocavallo cheese inoculated with *S. thermophilus*, *Lb. fermentum* CCS12BT, and *Lb. paracasei* subsp. *paracasei* CB9CT; CACIO6CT, Caciocavallo cheese inoculated with *S. thermophilus* and *Lb. paracasei* subsp. *paracasei* CACIO6CT; CCS12BT + CACIO6CT, Caciocavallo cheese inoculated with *S. thermophilus*, *Lb. fermentum* CCS12BT, and *Lb. paracasei* subsp. *paracasei* CACIO6CT. Different letters indicate significantly different ($P < 0.05$) mean values.

The CB9CT and control cheeses were subjected to the triangle test (ISO 4120:2004 standard protocol), in order to assess whether these two cheeses had different sensory traits. For this purpose, 42 assessors were recruited. According to the statistical table, Table A.1, annexed to the ISO 4120:2004 standard protocol, by inputting 42 assessors and by use of an α-risk level of 0.05, at least 20 correct responses are needed to conclude that a perceptible difference exists. Nineteen recruited assessors perceived differences between the tested cheeses (data not shown). This result indicates that no difference exists between the sensory traits of the CB9CT and the control cheeses.

DISCUSSION

Accumulation of BAs in cheese and other foods is a matter of public health concern (11). Cheeses, especially those made with raw milk, are complex ecosystems populated by microbes with diverse metabolic abilities (55). In the last few years, particular interest in the role of the cheese microbiota on BA formation and/or degradation has deservedly been shown to prevent or decrease the accumulation of such compounds in dairy products (27).

This study first screened a large number of presumptive autochthonous lactic acid bacteria (665 isolates) for the presence/absence of genes encoding enzymes that synthesize BAs. Currently, PCR-based methods, which aim at detecting genes responsible for BA formation, are regarded as the most suitable (34). Under the experimental conditions used in this study, a marked correlation between the presence of these genes and the capacity to decarboxylate precursor amino acids (His, Tyr, Lys, and Orn), thus producing the corresponding BA (histamine, tyramine, cadaverine, and putrescine), was found. This result strengthens the validity of the genetic approach used. Recently,

Ladero et al. (18) reported that almost all the lactic acid bacteria that possess BA-related genes synthesize BAs at the phenotypic level.

In this study, the highest percentage of isolates not producing BAs was roughly identified in cheeses (e.g., Caciocavallo Podolico Dauno, Cacioricotta, and Vastedda della Valle del Belìce PDO) which harbored low total levels (8 to 64 mg kg^{-1}) of BAs (cadaverine, histamine, putrescine, and tyramine) (14). The selection of starters, mainly to be used in form of adjunct cultures, which are unable to synthesize BAs could be an effective strategy for preventing the accumulation of BAs in cheeses (6). On the basis of genetic and phenotypic data, 431 isolates not producing BAs were studied for their capacity to grow on mCDM containing a BA. This method was successfully used by other researchers (27). Under this condition, 94 isolates were able to degrade at least one BA. Bacteria capable of growing in the presence of BAs were isolated from cheeses ripened for both a short period of time (e.g., Vastedda della Valle del Belìce PDO) and a long period of time (e.g., Tuma Persa), thus almost excluding a correlation between the degradation of BAs with the length of ripening (14). The number of isolates that degraded 2-phenylethylamine and tryptamine was lower than the number showing the capacity to degrade histamine and tyramine. Overall, cheeses from which the amine-degrading lactic acid bacteria were isolated showed lower concentrations of 2-phenylethylamine and tryptamine than histamine and tyramine (14). This would suggest that bacteria are induced to metabolically use BAs when these compounds reach a threshold value in cheese. The mono-AO of *Escherichia coli* is induced by the presence of tyramine in the medium (56). Overall, the ability to convert BAs into compounds that may be used as a carbon, nitrogen, or energy source (57, 58) would provide a metabolic advantage in nutrient-poor ecosystems, such as ripening cheese.

All the 94 isolates degrading BAs were typed by RAPD-PCR, and 78 strains were detected. Each of them was identified to the species level by partial sequencing of the 16S rRNA gene. First, this study showed that strains belonging to diverse species of lactic acid bacteria (*E. lactis*, *Lb. parabuchneri*, *Lb. paraplantarum*, *Lc. lactis*, *Ln. lactis*, *Ln. mesenteroides*, *S. gallolyticus*, *S. thermophilus*, *W. paramesenteroides*) were able to degrade BAs. In addition, strains belonging to *Lb. casei*, *Lb. rhamnosus*, and *P. pentosaceus* showed the potential to degrade BAs, in agreement with the findings of previous studies (19, 20, 22, 59).

To date, amine-degrading activities were attributed exclusively to AOs. Overall, amine-degrading bacteria are used to decrease BAs in foods, but the exact mechanism of action remains unknown (3, 19, 27, 60). Monoamine and diamine oxidases were reported to catalyze the detoxifying oxidation of BAs in the gastrointestinal tract of higher organisms (21). Moreover, AOs were identified in *Arthrobacter crystallopoietes*, *Candida boidinii*, *Klebsiella aerogenes*, *Micrococcus rubens*, *Sarcina lutea*, and *Rhodococcus erythropolis* (21, 61–63). Within lactic acid bacteria, the amine-degrading activity of *Lb. plantarum* J16 (alias, CECT 8944) was attributed to a protein annotated as laccase (EC 1.10.3.2) (64). In this study, *Lb. paracasei* subsp. *paracasei* CB9CT, which showed the highest activity toward histamine, was subjected to purification of its amine-degrading enzyme. The protein responsible for amine degradation was purified and identified to be a multicopper oxidase. The biological role(s) of multicopper oxidases in bacteria has been poorly elucidated (65–68). A gene coding for a protein that was similar to the laccase (a subtype of multicopper oxidase) of *Lb. plantarum* J16 (64) was found in the genome of the histamine- and tyramine-degrading bacterium *Lb. casei* 5b (69). The laccase of *Lb. plantarum* J16 was cloned and expressed in *E. coli*, and the recombinant enzymes catalyzed the oxidation of histamine, putrescine, and, especially, tyramine (64).

On the basis of the primary sequence of the multicopper oxidase of *Lb. paracasei* subsp. *paracasei* CB9CT, primers targeting the corresponding gene were designed and used in PCR experiments. The nucleotide sequence of the PCR product showed a high degree of similarity with the multicopper oxidases of two strains of the *Lb. casei* group. Among the other strains tested, only *Lb. fermentum*, *Lb. paraplantarum*, and *P. pentosaceus* gave PCR products whose sequences were highly similar to those of multicopper oxidase genes. It may be hypothesized that the capacity of other lactic acid bacterial strains (strains belonging to genera such as *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Weissella*) to use BAs could be due to different enzymes. Alternatively, the absence of PCR amplification could be due to the high specificity of the designed primers, which did not allow alignment with the target genes.

Lb. paracasei subsp. *paracasei* CB9CT and *Lb. paracasei* subsp. *paracasei* CACIO6CT were successfully used as a single adjunct starter for decreasing the concentrations of histamine and tyramine in industrial Caciocavallo-type cheese. Compared to the results for the control, the cheeses containing the adjunct starters showed a higher cell density of presumptive mesophilic lactobacilli throughout ripening, suggesting that the selected amine-degrading strains survived the stretching phase. All the experimental cheeses, including the control, showed concentrations of individual BAs below the safety threshold of 100 mg kg^{-1} (70). The gross composition and pH of the cheeses to which the amine-degrading strains were added did not significantly differ from those of the control cheeses. Furthermore, no significant difference in sensory traits between the CB9CT and control cheeses was perceived. Previously, the concentration of histamine and tyramine in a Cabrales-like minicheese was lowered when two strains of *Lb. casei*, which were isolated from Zamorano cheese, were used (27).

Interest in the characterization of nonstarter lactic acid bacteria (NSLAB) from artisanal products to be used as tailored cultures for making dairy products with protected geographic indication (PGI) status is increasing. *Lb. paracasei* is a common member of the NSLAB population in cheeses (71). Given the concerns over the presence of BAs in traditional cheeses, this study showed that selected strains of *Lb. paracasei* could be also used to decrease the concentrations of histamine and tyramine in cheeses. This would result in increased cheese safety, while it would maintain the typical sensorial traits of traditional cheeses.

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R.G. performed all molecular analyses and wrote the manuscript; M.D.A. discussed the results and wrote the manuscript; L.S. directed the experimental phases; S.F. performed the enzyme extraction and purification and analyzed the experimental cheeses; R.G. performed the selection of the lactic acid bacteria; F.M. directed the experimental phases and wrote the manuscript; G.M. discussed the results; M.G. ideated the study.

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