Food Bioscience 53 (2023) 102582

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Journey to the morpho-textural traits, microbiota, and volatilome of *Ciauscolo* PGI salami

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ARTICLE INFO

Keywords:
Fermented sausages
Metataxonomic analysis
Latilactobacillus sakei
Acidification performances
Enzymatic activity

ABSTRACT

Two independent manufacturing batches of naturally fermented *Ciauscolo* PGI salami were studied from the day of production to the end of ripening (20 days). The microbiota compositional shift of samples was studied through culture-dependent and metataxonomic analysis. Analyses of physico-chemical, morpho-textural, and volatilome traits were also carried out. *Latilacobacillus sakei* was the dominant species from t_5, together with *Dellaglioa algida* and *Leuconostoc carnosum*. Among eumycetes, *Cladosporium cladosporioides*, *Debaryomyces hansenii*, *Kurtzmaniella zeylanoides*, and *Malassezia restricta/globosa* were the most abundant taxa occurring in all samples. Candidate starter or adjunct cultures of *Lat. sakei* were isolated and characterized for: i) acidification performance; ii) enzymatic activity; iii) production of exopolysaccharides; iv) production of bacteriocins; v) quantification of the *hdcA* gene of Gram-positive bacteria. The 44 *Lat. sakei* cultures showed a suitable acid production capacity, together with the capability to cope with the main environmental stresses occurring in fermented sausages. For most of the isolates, strong aminopeptidase activity (leucine arylamidase and the valine arylamidase) was observed. Moreover, the majority of isolates showed the *in vitro* production of sucrose-dependent exopolysaccharides. No *Lat. sakei* isolates positive for the *hdcA* gene were observed. Based on these evidences, a few candidate starter or adjunct cultures, with potential use for product safety and quality improvement, were highlighted, namely *Lat. sakei* C5, C7, C11, C31, C45, C48, C53, C55, and C60. In the analyzed *Ciauscolo* PGI samples, 53 volatile substances were fully or tentatively identified; among these, spices-derived components (black pepper and garlic cloves) were constantly detected throughout the whole ripening time.

1. Introduction

The preservation of meat through drying and fermentation represents the most ancient method to overcome meat decay, as already told in the Homer's Odyssey, ca. 900 B.C., and in some historical texts dating back to the old Roman Empire (Zeuthen, 1995). Nowadays, those that are called charcuterie products represent food delicacies that are highly appreciated by consumers, since they are produced in accordance with ancient traditions. Among charcuterie products, fermented sausages are usually manufactured using minced swine meat (or meat from other animal species as chicken or lamb) and lard, added with salt and spices, and stuffed into animal casings. Sugars, preservatives, antioxidants, and microbial starter cultures can also be added to the meat batter (Cruxen et al., 2019). Then, sausages are left to ferment at specific environmental conditions (temperature and relative humidity) that vary according to the production process.

During fermentation, the sausage is subjected to physico-chemical modifications that are the result of the activity of native meat enzymes and microorganisms (Belleggia et al., 2022a). Such modifications contribute to the firmness, cohesiveness, and safety of fermented sausages (Cruxen et al., 2019).

In fermented sausages, the microbial succession occurring from the stuffing of the meat batter till the end of ripening produces the transformation of the raw meat into the end product. In more detail, as soon

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https://doi.org/10.1016/j.fbio.2023.102582
Received 2 February 2023; Received in revised form 21 March 2023; Accepted 22 March 2023
Available online 22 March 2023

as the meat batter is stuffed into the natural or artificial casing, the naturally occurring adventitious microbiota (e.g., Enterobacteriaceae, *Pseudomonas*, *Campylobacter*, *Psychrobacter*, and *Brochothrix*) is progressively substituted by pro-technological microorganisms as lactic acid bacteria, coagulase-negative cocci, and eumycetes (Belleggia et al., 2020a).

Lactic acid bacteria are among the key pro-technological microorganisms involved in the biochemical, microbiological, and sensory modifications occurring during the production of fermented sausages (Zdolec et al., 2022). Lactic acid bacteria can naturally be present as minority fraction in the raw materials, or they can deliberately be added to the meat batter as starter or adjunct cultures. In fermented sausages, lactic acid bacteria are primarily responsible for the acidification of the product; moreover, they can exert other biological activities that include the production of bacteriocins and exopolysaccharides (EPS), with positive affect on safety and sensory attributes of the product (Belleggia et al., 2022b). Of note, the metabolic activity of lactic acid bacteria can every so often produce unwanted substances, as biogenic amines (e.g., histamine and tyramine), with negative impact on the safety of fermented sausages (Anderegg et al., 2020; Fong et al., 2021). Regarding histamine, this biogenic amine is produced through the bacterial decarboxylation of the amino acid histidine and is the causative agent of the so-called scombroid poisoning. Decarboxylation of histidine carried out by Gram-positive bacteria is determined by the activity of histidine decarboxylase encoded by a gene cluster that includes the *hdcA* gene (Diaz et al., 2016).

Although the use of lactic acid bacteria as starters or adjuncts for the production of fermented sausages is well established, the selection and characterization of new cultures with biotechnological and safety advantages is still needed (Zdolec et al., 2022). To this end, naturally fermented sausages could represent a source of microbial biodiversity for the selection of lactic acid bacteria with pro-technological features to be used for product improvement (Zdolec et al., 2022).

In Central Italy, a great number of naturally fermented sausages is produced in accordance with ancient traditions, with *Ciauscolo* PGI salami being recognized as one of the most well-known and appreciated products of the Marche Region (Central Italy). *Ciauscolo* PGI salami obtained the Protected Geographical Indication (PGI) status according to Commission Regulation (EC) No 729/2009 of 10 August 2009 (Regulation (EC) No 729/2009). The name *Ciauscolo*, also referred to as *Ciavuscolo* or *Ciabuscolo*, originates from the Latin words "*ciabusculum*" or "*cibusculum*" that were used to describe a little portion of food or snack that farmers were used to consume in tiny quantities during breaks and between main meals.

Ciauscolo PGI salami consists of a mixture of pork meat resulting from the following cuts used in descending order: belly, up to a maximum of 70% (w w^{-1}); shoulder, up to a maximum of 40% (w w^{-1}); trimmings of ham and loin, up to a maximum of 30% (w w^{-1}). The main physical characteristic of *Ciauscolo* PGI salami is softness up to spreadability.

To produce *Ciauscolo* PGI salami, the meat cuts are minced using a 2–3 mm plate, together with salt, ground black pepper, wine, and crushed garlic. The addition of sugars (lactose, dextrose, fructose, or sucrose) as well as of L-ascorbic acid, sodium ascorbate, potassium nitrate is allowed. Once prepared, the meat batter is stuffed into pork or bovine intestine casings and ripened for at least 15 days. The final pH of *Ciauscolo* PGI salami must be higher than or equal to 4.8, with minimum protein content attesting at 15% (w w^{-1}) and fat content comprised between 32% and 42% (w w^{-1}). According to the production disciplinary, the maximum water/protein ratio allowed is 3.10, whereas the maximum fat/protein ratio allowed is 2.80. The weight of the end product ranges from 400 to 2500 g, and the diameter ranges between 4.5 and 10 cm. *Ciauscolo* PGI salami has a cylindrical shape with a length comprised between 15 and 45 cm. The slice of *Ciauscolo* PGI salami is pink, uniform, homogeneous, and free from rancid fractions. The smell of this fermented sausage is delicate, aromatic, and spicy, whereas the taste is savory and delicate, but not acidic. The minimum load of lactic acid bacteria that must be present in the end product is higher than 7 log colony forming units (cfu) g^{-1}. *Ciauscolo* PGI salami can only be manufactured in the geographical area covered by four (Ancona, Ascoli Piceno, Fermo, and Macerata) out of the five provinces of the Marche Region.

Although a few studies already dealt with the microbiology of *Ciauscolo* PGI salami (Aquilanti et al., 2007; Belleggia et al., 2020a; Federici et al., 2014; Silvestri et al., 2007), to the authors' knowledge no research on the characterization of pro-technological traits of autochthonous lactic acid bacteria isolated from this peculiar food matrix has been performed, yet. Similarly, scarce data on its volatilome are available in the scientific literature. Hence, in the present study, different selective growth media and a metataxonomic approach were applied to study the microbiota compositional shift occurring in spontaneously fermented *Ciauscolo* PGI salami manufactured by an artisanal producer located in the Marche Region. Lactic acid bacteria with potential pro-technological features (starter or adjunct cultures) were also isolated. Moreover, the analysis of volatile organic compounds (VOCs) via Headspace Solid-Phase Microextraction-Gas Chromatography/Mass Spectrometry (HS-SPME-GC/MS) analysis was also carried out. Finally, a pool of isolates ascribed to *Latilactobacillus sakei* were characterized for: i) acidification activity; ii) key enzymatic activities; iii) the production of EPS; iv) quantification of the *hdcA* gene of Gram-positive bacteria encoding for histidine decarboxylase.

As reported by the EFSA BIOHAZ Panel (EFSA BIOHAZ Panel EFSA Panel on Biological Hazards, 2018), ready-to-eat foods, including fermented sausages, might represent a risk for the presence of *Listeria monocytogenes* (Petruzzelli et al., 2010), hence, the *Lat. sakei* isolates were also tested for production of bacteriocins against *Listeria innocua*, utilized as surrogate for *L. monocytogenes* (ANSES, 2019).

2. Materials and methods

2.1. Ciauscolo PGI salami production

Two independent manufacturing batches (marked as batch 1 and batch 2) of *Ciauscolo* PGI salami were analyzed. Both batches were manufactured by an artisan producer located in the geographical area of production (Macerata province of the Marche Region), in accordance with the *Ciauscolo* PGI salami production disciplinary. The first batch was produced in early spring, whereas the second in in late spring of the same year. According to the disciplinary for production of *Ciauscolo* PGI salami, the meat batter was prepared using the following swine cuts: shoulder 25% (w w^{-1}), belly (including fat) 40% (w w^{-1}), loin 10% (w w^{-1}), and ham 21% (w w^{-1}). Moreover, salt (Italkali, Palermo, Italy) 2.7% (w w^{-1}), ground black pepper (Dama, Rezzato, Italy) ~0.4% (w w^{-1}), ground garlic (Dama, Rezzato, Italy) ~0.1% (w w^{-1}), white wine (Rocchi, Belforte del Chienti, Italy) (20 mL kg^{-1}), and potassium nitrate (E252) (Tec-Al, Traversetolo, Italy) 0.05% (w w^{-1}) were also added. No starter cultures were added.

For each batch a total of 100 kg of meat batter was processed. The meat batter was minced twice with a 3 mm plate and stuffed into swine bowels previously washed in a mixture of water and white wine vinegar (Lodovici, Savignano sul Panaro, Italy) 50% (v v^{-1}). The drying was performed for 5 days under the following conditions: progressive temperature decrease, from 20 °C to 14 °C, and concomitant progressive relative humidity (R.H.) decrease from 99% to 60%. Ripening of the sausages was carried out at 15 °C and 75% R.H. for 15 days.

For each batch, fermented sausages approximately weighing 750 g, with 26 cm length and 5.5 cm width, were produced (Fig. 1). Collection of samples was performed at 0, 5, 10, and 20 days; for each ripening time and batch, three fermented sausages (for a total of 24 sausages) were collected. Samples were aseptically collected using sterile instruments and bags (Nasco Whirl-Pak Easy-To-Close Bags, Fisher Scientific Italia, Rodano, Italy). The samples were rapidly refrigerated and analyzed

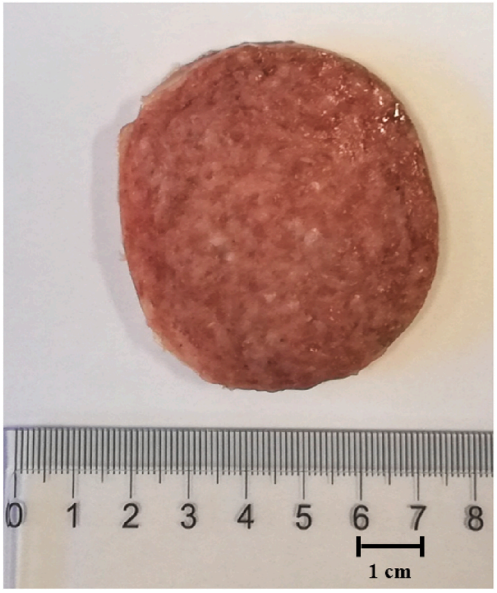


Fig. 1. Slice of ready-to-eat *Ciauscolo* PGI salami.

during the same day of collection.

2.2. Physico-chemical analyses

A pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy) was used to determine pH values of *Ciauscolo* PGI salami samples through direct insertion of the probe into the food matrix. The data, in duplicate for each sample, were reported as mean ± standard deviation.

Total titratable acidity (TTA) was determined on 10 g of sample homogenized in 90 mL of distilled water, adjusted to a pH value of 8.3 with a 0.1 N solution of NaOH (Merck KGaA, Darmstadt, Germany) used as a titrant. Percentage (%) TTA of lactic acid equivalents was calculated as previously reported by Rampanti et al. (2023). TTA% was reported as mean ± standard deviation of two replicates.

The amounts of acetic acid and lactic acid were assessed using Acetic Acid (Acetate Kinase Manual Format) test kit (Megazyme, Bray, Ireland) and D-/L-Lactic Acid (D-/L-Lactate) (Rapid) test kit (Megazyme). The data, in duplicate for each sample, were expressed as g 100 g^{-1} of sample and reported as mean ± standard deviation.

The water activity (a_w) was assessed in accordance with the ISO 21807:2004 standard method using an Aqualab 4 TE apparatus (Meter Group, Pullman, USA). The data, in duplicate for each sample, were reported as mean ± standard deviation.

Color measurements were performed using a Chroma Meter CR-200 (Minolta, Osaka, Japan) with a D65 illuminant. Color was determined on 2 cm thick slices according to CIE L*a*b* system (L*, lightness; a*, redness/greenness; b*, blueness/yellowness) (Belleggia et al., 2022b). Visual changes during ripening of the sausages were evaluated by longitudinally cutting them (7 mm thickness) and imaging the cross sections with a ENVY 6200 Series scanner (HP, Palo Alto, CA, USA) (Dreher et al., 2021).

Cylindrical specimens of sausages (height: 15 mm, diameter: 20 mm) were excised and then uniaxially compressed twice with a CT3-4500 texture analyzer (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) equipped with a 36 mm diameter cylindrical probe (mod. TA-AACC36) at 1.5 mm s^{-1} using a non-destructive deformation (40%) (Dreher et al., 2021). Specimens were positioned between the load cell and the fixture base table of the instrument. A 4500 g load cell was used.

2.3. Viable counts

Viable counts were performed by mixing 10 g of each sample with 90 mL of sterile peptone water (1 g L^{-1} of bacteriological peptone) homogenized using a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 3 min at 260 rpm. After homogenization, ten-fold serial dilutions were prepared, and viable counts of the following microbial groups were evaluated: i) lactic acid bacteria on De Man Rogosa and Sharpe (MRS) agar (Merck), added with 250 mg L^{-1} of cycloheximide (Merck) (250 mg L^{-1}) to inhibit the growth of eumycetes, with incubation at 37 °C for 48–72 h; ii) coagulase-negative cocci on Mannitol Salt Agar (MSA) (Liofilchem, Roseto degli Abruzzi, Italy), with incubation at 37 °C for 48–72 h; iii) Enterobacteriaceae on Violet Red Bile Glucose Agar (VRBGA) (VWR Chemicals, Radnor, PA, USA), with incubation at 37 °C for 24 h; iv) eumycetes on Rose Bengal Chloramphenicol Agar (VWR Chemicals), with incubation at 25 °C for 72–96 h. The results of two biological and three technical replicates were expressed as the log of colony-forming units (cfu) per gram of sample and reported as mean ± standard deviation.

Finally, a miniVIDAS apparatus (bioMérieux, Marcy l'Etoile, France) was used to assess the presence/absence of *Listeria monocytogenes* and *Salmonella* spp. through the enzyme-linked fluorescent assay (ELFA) method, in accordance with the AFNOR BIO 12/11-03/04 and AFNOR BIO 12/16-09/05 standard methods, respectively (Haouet et al., 2017).

2.4. Real-time PCR analysis for the detection of botulinic toxins genes

The presence/absence of *Clostridium botulinum* was assessed via multiplex real-time PCR as already described by Belleggia et al. (2020a). *C. botulinum* was analyzed in accordance with the multiplex real-time PCR method of the Italian National Reference Centre for botulism (http://old.iss.it/binary/cnrb/cont/CNRB31.010.pdf) for the detection of the target genes *bont/A, bont/B, bont/E, bont/F, and* 4gyrB *(CP*) that encode for botulinic toxins.

Briefly, 25 g of *Ciauscolo* PGI salami sample were blended in 225 mL Triptone Peptone Glucose Yeast extract (Microbiol Diagnostici, Uta, Italy), incubated in anaerobiosis at 30 °C for 96 h and then extracted with 6% Chelex-100 (BioRad Laboratories, Hercules, CA, USA). The amplification was carried out using the Kit QuantiTect multiplex No Rox (Qiagen, Hilden, Germany) in the Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA) thermal cycler; the primers and probes nucleotide sequences were those already reported by Belleggia et al. (2020a). Two different Master mixes were used concurrently, with the following thermal profile: 1 cycle at 95 °C for 15 min followed by 40 cycles at 94 °C for 30 s and 56 °C for 90 s.

2.5. RNA extraction and cDNA synthesis

For each sample homogenate (dilution 10^{-1}), prepared as reported above, 1.5 mL aliquot was centrifugated at 13,148×*g* for 10 min using a 5415C Centrifuge (Eppendorf, Hamburg, Germany). RNA*later* Stabilization Solution (Ambion, Foster City, CA, USA) was used to protect the resulting cell pellets that were subsequently stored at −80 °C. QuickRNA MiniPrep kit (Zymo Research, Irvine, CA, USA) was then used to extract the total microbial RNA from the cell pellets in accordance with manufacturer's instructions. PCR amplification with universal prokaryotic primers (27 f, 1495 r) (Weisburg et al., 1991) was carried out to check for the presence of residual DNA. SensiFAST cDNA Synthesis Kit for RT-qPCR (Bioline, London, UK) was used to synthesize cDNA according to manufacturer's instructions.

2.6. Amplicon target sequencing and bioinformatic analysis

The cDNA was used as template to amplify the V3–V4 region of the 16S rRNA gene for bacteria (Klindworth et al., 2013), whereas the D1 domain of the 26S rRNA gene of Large Ribosomal Subunit (LSU) was

targeted for fungi (Mota-Gutierrez et al., 2019). PCR templates were then purified and processed following the Illumina metagenomic pipeline (Illumina, San Diego, CA, USA). Sequencing was performed with a MiSeq instrument (Illumina) in a 2X250bp configuration.

A total of 400,802 and 535,306 raw-reads were produced by 16S and 26S amplicon-based sequencing, respectively. To obtain Amplicon Sequence Variants (ASVs) the raw-reads were analyzed with *DADA2* package (Callahan et al., 2016) in R environment (R version 4.1.1; http://www.r-project.org). The pipeline described by Botta et al. (2022) was applied to filter and merge raw-reads: 157,311 paired-end bacterial reads (19,664 reads/sample) and 270,743 paired-end fungal reads (45,124 reads/sample) passed the filtering parameters and were used to construct ASVs tables.

Taxonomy was assigned with a 99% sequence similarity through Bayesian classifier method (Wang et al., 2007) by matching bacterial ASVs to the 2021 release (version 138.1) of Silva prokaryotic SSU reference database (https://zenodo.org/record/4587955#.YObFvhMzZRE). Fungal ASVs taxonomy was assigned at 99% against an internal database of 26S rRNA (Mota-Gutierrez et al., 2019). Taxonomy assignment for 16S and 26S was double checked by using BLASTn suite (https://blast.ncbi.nlm.nih.gov).

Fungal and bacterial ASVs were aligned with *DECIPHER* package and two unrooted phylogenetic tree were constructed with *phangorn* package (Schliep, 2011; Wright, 2016). Alpha diversity metrics and weighted UniFrac beta-diversity distance were calculated with *phyloseq* and *picante* packages (Kembel et al., 2010; McMurdie and Holmes, 2013): rarefaction limit was set to the lowest number of sequences/sample.

2.7. GC-MS analysis of volatile components

Two g of fresh salami were weighed in a 10 mL screw cap septum vial, then the vial was equilibrated at 40 °C for 15 min in a thermostatic bath. The static headspace was sampled by a 65 μm PDMS/DVB SPME fibre (Supelco, Bellefonte, PA, USA) for 15 min. Chromatographic separation of volatiles was performed by a fused silica capillary column ZB-5 (30 mL, 0.25 mm ID, 0.25 μm FT) (Phenomenex, Torrance, CA, USA) mounted on a Varian 3900 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA); the injector was operating in splitless mode for 0.1 min at a constant temperature of 250 °C; oven temperature was increased from 40 °C to 220 °C at a rate of 6 °C min^{-1}, then held at the final temperature for 5 min; carrier gas (He) was set at constant flow mode (1.0 mL/min). The gas chromatograph was coupled to an ion trap mass detector Saturn 2100T (Varian Analytical Instruments): the trap and the transfer line were set at 200 °C and 220 °C, respectively; electron impact (70 eV) mass spectra were acquired in the mass range of 31–250 amu.

Volatile compounds were identified by matching mass spectral data collected in the NIST/EPA/NIH Mass Spectral Library (Version 2.0a, build July 1, 2002; National Institute of Standards and Technology) and Kovats Retention Indexes (RIs) available in the public access database Pubchem (https://pubchem.ncbi.nlm.nih.gov/). A C8–C20 normal alkanes mixture (Sigma-Aldrich, St. Louis, MO, USA) was used to calculate RIs. An automated spreadsheet (Lucero et al., 2009) was used for simplifying the calculation of RIs ok unknown components and speeding up the comparison with published indexes.

2.8. Isolation and characterization of Lat. sakei

2.8.1. Isolation and identification

As previously described, colonies of lactic acid bacteria grown on MRS agar (Merck) added with cycloheximide (Merck) were randomly selected and subsequently sub-cultured to purity under the same conditions. Isolates were obtained from all the three samples collected at each sampling time and batch. The obtained isolates were then stored at −80 °C.

Prior to further analysis, lactic acid bacteria were thawed and sub-cultured twice on MRS agar (Merck) at 37 °C for 48 h. DNA was extracted from the lactic acid bacteria isolates according to Osimani et al. (2015) and its purity and quantity was verified with a NanoDrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). DNAs were standardized to a final concentration of 100 ng μL^{-1} and subjected to PCR in a My Cycler Thermal Cycler (BioRad) using the universal prokaryotic primers 27f and 1495r, as described by Osimani et al. (2015). The amplification was verified by electrophoresis in 1.5% (w v^{-1}) agarose gel in 0.5X Tris/Borate/EDTA (TBE) buffer (VWR Chemicals) containing 0.5 μg mL^{-1} GelRed® Nucleic Acid Gel Stain, 10,000X in water (Biotium, San Francisco Bay Area, USA). The electrophoretic run included the HyperLadder™ 1 kb (Meridian Bioscience, Cincinnati, OH, USA) as molecular weight standard and was carried out at 75 V for 3.5 h and visualized under UV light. The amplicons were then shipped to Genewiz (Takaley, UK) for their purification and sequencing.

The raw sequences were analyzed with UCHIME2 software tool to uncover chimeras (Edgar, 2016) and trimmed to remove NNNs and misleading data from the terminations. Afterwards, a BLAST search was exploited to compare the obtained sequences with 16S rRNA sequences of type strains from GenBank DNA database (http://www.ncbi.nlm.nih.gov/). The sequences of the lactic acid bacteria cultures were finally submitted to GenBank DNA database to acquire the respective accession numbers.

2.8.2. Acidification in synthetic medium

Lat. sakei isolates were sub-cultured twice in MRS broth (Merck) incubated at 30 °C for 18 h (Wen et al., 2021). The cultures were centrifuged at 1610×*g* for 5 min using a Rotofix 32A centrifuge (Hettich, Milano, Italy) and the pellets washed with sterile physiological solution (0.9% w v^{-1}) prior to resuspension in the same diluent. The concentration of bacterial cells in the suspensions was determined using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) by measuring the optical density (OD) at 600 nm. All the isolates were inoculated to 8 log cfu mL^{-1} in 10 mL of: i) MRS broth (Merck); ii) MRS broth (Merck) added with 150 mg kg^{-1} of E250 (sodium nitrite) (Tec-Al); iii) MRS broth (Merck) added with 150 mg kg^{-1} of E252 (potassium nitrate) (Tec-Al); iv) MRS broth (Merck) added with 3% (w v^{-1}) of NaCl (Italkali); v) MRS broth (Merck) added with 150 mg kg^{-1} of E250 (Tec-Al), 150 mg kg^{-1} of E252 (Tec-Al), and 3% (w v^{-1}) of NaCl (Italkali). The pH values of the media were measured prior to inoculation (t_0) and after incubation at 30 °C for 4 and 24 h.

2.8.3. Semi-quantitative assessment of enzymatic activities

The semi-quantitative micromethod API® ZYM (bioMérieux) was used for the assessment of key enzymatic activities of the selected pool of lactic bacteria in accordance with manufacturer's instructions. Each API® ZYM (bioMérieux) strip is composed of 20 cupules containing synthetic substrates that are inoculated with the microorganism to be tested in a water suspension, this latter used to rehydrate the enzymatic substrates. The 20 cupules are designed to evaluate the activity of the following enzymes: 1 – control; 2 - alkaline phosphatase; 3 - esterase (C 4); 4 - esterase lipase (C 8); 5 - lipase (C 14); 6 - leucine arylamidase; 7 - valine arylamidase; 8 - cystine arylamidase; 9 - trypsin; 10 - alpha-chymotrypsin; 11 - acid phosphatase; 12 - naphthol-AS-BI-phosphohydrolase; 13 - alpha-galactosidase; 14 - beta-galactosidase; 15 - beta-glucuronidase; 16 - alpha-glucosidase; 17 - beta-glucosidase; 18 - N-acetyl-ß-glucosaminidase; 19 - alpha-mannosidase; 20 - alpha-fucosidase. The metabolic end-products produced during the incubation period were detected through coloured reactions revealed by the addition of reagents.

Briefly, a suspension in API Suspension Medium (bioMérieux) (2 mL) of each isolate grown on MRS agar (Merck) was prepared according to a turbidity of 5–6 McFarland (bioMérieux). Sixty-five μL of the obtained suspension were used for the inoculation of each cupule of the API® ZYM (bioMérieux) strips with incubation for 4 h at 37 °C. After incubation, 1 drop of ZYM A reagent (bioMérieux) and 1 drop of ZYM B

reagent (bioMérieux) were added to each cupule until colour development (at least 5 min). For each cupule, a value ranging from 0 to 5 was assigned, corresponding to the colour developed: 0 corresponding to a negative reaction, 5 to a reaction of maximum intensity and 1, 2, 3, or 4 were intermediate reactions depending on the level of intensity (3, 4, or 5 being considered as positive reactions).

2.8.4. Assessment of antimicrobial activity

The antimicrobial activity of the selected pool of isolates ascribed to *Lat. sakei* was assessed by following the agar well diffusion assay, described by Parente et al. (1995). Briefly, molten Brain Heart Infusion (BHI) soft agar (0.75% agar) (VWR Chemicals) was inoculated (2%, v v^{-1}) with the indicator microorganism *Listeria innocua*. Twenty mL of the inoculated medium were poured into 90 mm Petri dish (VWR Chemicals) and left to solidify. A cone of a 200 μL sterile tip (VWR Chemicals) was utilized to create wells of ~50 μL capacity on BHI soft agar (VWR Chemicals). Previously, lactic acid bacteria were sub-cultured twice in MRS broth (Merck) at 37 °C for 48 h. The broth cultures were then added with 0.1 N NaOH (AppliChem, Darmstadt, Germany) solution to reach pH 7.0 to neutralise the organic acids produced during the bacterial growth. A filtration step on sterile PES membrane filter of 0.22 μm pore size (Laboindustria S.p.A., Padova, Italy) followed. For each lactic acid bacteria isolate, 4 wells were formed on BHI soft agar (VWR Chemicals), each containing: (i) 50 μL of the sub-cultured suspension; (ii) 50 μL of the neutralised suspension adjusted to pH 7.0; (iii) 50 μL of the filtered neutralised suspension; (iv) 50 μL of sterilised water as a negative control. Afterwards, the Petri dishes (VWR Chemicals) were incubated at 37 °C for 24 h and examined for the presence of zones of inhibition. In the case of positive results (presence of inhibition halo), 3 spots of 5 μL each of pepsin (Fluka™, Honeywell, Morristown, USA), trypsin (Fluka™) or Pronase (Merck) were set along the circumference of the inhibition zone to evaluate the protein nature of the antimicrobial. The Petri dishes were further incubated at the same conditions. The antimicrobial activity of lactic acid bacteria isolates due to the synthesis of bacteriocins was confirmed by the formation of crescents.

2.8.5. In-vitro EPS production

The selected pool of isolates ascribed to *Lat. sakei* was screened for EPS production, based on the method already reported by Hilbig et al. (2019) with some modifications. In more detail, the isolates were retrieved from cryo-protective suspensions and sub-cultured twice on MRS broth (Merck) at 37 °C for 48 h. The EPS production was visually observed by adding aliquots (5 μL) of each bacterial culture on the following solid media: (i) MRS agar (Merck) supplemented with sucrose (Serva, Heidelberg, Germany) (80 g L^{-1}) to promote the synthesis of homopolysaccharides (HoPS); MRS agar (Merck) supplemented with yeast extract (VWR Chemicals) (10 g L^{-1}), meat extract (VWR Chemicals) (10 g L^{-1}), galactose (VWR Chemicals) (20 g L^{-1}), and lactose (Carlo Erba Reagents, Cornaredo, Italy) (20 g L^{-1}) to promote the synthesis of heteropolysaccharides (HePS). After an incubation period of 48 h at 30 °C, the colonies were classified as positive whenever presenting a mucoid aspect (visible shiny and slimy appearance) or a ropy consistence (able to produce detectable filaments by using a sterile toothpick). For each isolate, the analyses were carried out in duplicate.

2.8.6. Quantification of the hdcA gene

The quantification of the *hdcA* gene in the *Lat. sakei* isolates was carried out in a CFX Connect Real-Time System machine (Biorad). The primers used in the qPCR reactions and the cycling conditions were set as previously described by Belleggia et al. (2021). The positive strain *Lactobacillus parabuchneri* DSM 5987 was used to create the standard curve. The analysis was carried out in three technical replicates for each sample, together with a blank, and the results were expressed as the mean of Log gene copies per g of sample ± standard deviation. Since histamine-producing Gram-positive bacteria contain a unique copy of the *hdcA* gene, the results were also expressed as Log cells per g of sample.

2.9. Statistical analysis

To assess statistical differences within sausage samples, the Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used by one-way analysis of variance (ANOVA). Tests were performed through JMP v11.0.0 software (SAS Institute Inc., Cary, NC).

Permutational Multivariate Analysis of Variance (PERMANOVA) of the metataxonomic data were performed and mycobiota/microbiota compositions were compared between the two batches by Wilcoxon rank sum test (*P* value adjustment method: FDR) in R environment.

Correlation between taxa (ASVs merged at highest taxonomic level achieved) and VOCs was performed by means of Spearman's rank correlation.

3. Results

3.1. Physico-chemical analyses

The results of physico-chemical analyses carried out on the analyzed *Ciauscolo* PGI samples are reported in Table 1.

In more detail, pH values ranged between 5.94 and 5.23, with samples collected at t_0 showing the highest values, irrespective of the production batch.

As for a_w, the detected values were comprised between 0.961 and 0.936, with samples collected from t_{10} to t_{20} showing statistically significant lower values.

TTA % ranged between 0.594 and 0.990; for this parameter, as expected, the highest % were detected in samples at the end of ripening, irrespective of the production batch.

Regarding organic acids, lactic acid content ranged between 0.368

Table 1
Physico-chemical parameters of *Ciauscolo* PGI salami during fermentation and ripening.

Batch	Sampling time (days)	pH	a_w	TTA %	Lactic acid (g $100g^{-1}$)	Acetic acid (g $100g^{-1}$)
1	t_0	5.70 ± 0.04^{a}	0.960 ± 0.001^{a}	0.594 ± 0.006^{c}	0.368 ± 0.027^{c}	0.015 ± 0.004^{c}
	t_5	5.44 ± 0.01^{b}	0.954 ± 0.001^{a}	0.693 ± 0.006^{bc}	0.559 ± 0.045^{b}	0.034 ± 0.009^{b}
	t_{10}	5.55 ± 0.00^{b}	0.941 ± 0.001 ^{b}	0.765 ± 0.038^{b}	0.607 ± 0.057^{b}	0.060 ± 0.008^{a}
	t_{20}	5.52 ± 0.01^{b}	0.940 ± 0.002 ^{b}	0.909 ± 0.025^{a}	0.684 ± 0.006^{a}	0.069 ± 0.002^{a}
2	t_0	5.94 ± 0.01^{a}	0.961 ± 0.001^{a}	0.594 ± 0.013^{c}	0.411 ± 0.003^{c}	0.014 ± 0.002^{c}
	t_5	5.39 ± 0.03^{b}	0.955 ± 0.001^{b}	0.756 ± 0.019^{b}	0.647 ± 0.009^{b}	0.033 ± 0.001^{b}
	t_{10}	5.23 ± 0.01^{b}	0.942 ± 0.001^{b}	0.891 ± 0.000^{b}	0.763 ± 0.002^{b}	0.025 ± 0.011^{bc}
	t_{20}	5.57 ± 0.06^{b}	0.936 ± 0.001^{c}	0.990 ± 0.013^{a}	0.901 ± 0.003^{a}	0.059 ± 0.031^{a}

Values are expressed as mean ± standard deviation.
Samples are grouped by batch and displayed according to time.
T_0, day of production; T_5, samples after 5 days of ripening; T_{10}, samples after 10 days of ripening; T_{20}, samples after 20 days of ripening.
For each batch, different letters in the same column indicate significant differences according to the Tukey–Kramer's (HSD) test (= 0.05).

and 0.901 g 100 g^{-1}, with the highest values recorded in samples at the end of ripening, irrespective of the production batch. Acetic acid content was comprised between 0.014 and 0.069 g 100 g^{-1}, with statistically significant higher values recorded at the end of ripening, irrespective of the production batch.

3.2. Morpho-textural properties of Ciauscolo PGI salami

The results of color measurements during ripening of the analyzed sausages are reported in Fig. 2.

In more detail, the lightness (L*) attested between 49.89 and 55.13 for batch 1, and between 54.03 and 62.33 for batch 2. For each batch, the values for this parameter showed no significant differences during the ripening of *Ciauscolo* PGI salami.

As for redness/greenness (a*), the detected values were comprised between 9.57 and 12.7 (batch 1), and between 9.87 and 12.64 (batch 1). In the two batches, the highest a* values were observed at t_5.

Finally, blueness/yellowness (b*) values were comprised between 1.44 and 2.69 (batch 1), and 1.64 and 2.75 (batch 2). No differences were observed for the b* values of the samples collected from batch 1, whereas, in samples from batch 2, the lowest b* values were observed at t_{10} and t_{20}.

The results of texture analysis are reported in Table 2.

In more detail, values for hardness were comprised between 8.23 and 13.80 N (batch 1), and 7.79 and 13.21 N (batch 2). In both the analyzed batches, the highest values for this texture parameter were detected at t_{20}.

As for cohesiveness, the detected values ranged between 0.33 and 0.48 (batch 1), and 0.28 and 0.48 (batch 2). Within the same batch, no statistically significant values were observed among samples.

Finally, springiness ranged between 3.60 and 6.35 mm (batch 1), and 3.10 and 6.25 mm (batch 2). The highest springiness values were observed at t_0 for samples form batch 1, and at t_{10} for samples from batch 2.

Table 2
Results of texture analysis of *Ciauscolo* PGI salami during fermentation and ripening.

Batch	Sampling time (days)	Hardness (N)	Cohesiveness	Springiness (mm)
1	t_0	8.23 ± 1.43^{b}	0.48 ± 0.01^{a}	6.35 ± 0.35^{a}
	t_5	11.67 ± 2.66^{ab}	0.33 ± 0.09^{a}	3.60 ± 0.28^{c}
	t_{10}	10.86 ± 0.39^{ab}	0.33 ± 0.06^{a}	5.15 ± 0.21^{b}
	t_{20}	13.80 ± 1.22^{a}	0.40 ± 0.02^{a}	4.25 ± 0.21^{bc}
2	t_0	7.79 ± 0.57^{b}	0.48 ± 0.06^{a}	5.40 ± 0.28^{ab}
	t_5	11.65 ± 0.31^{ab}	0.28 ± 0.04^{a}	3.10 ± 0.42^{c}
	t_{10}	12.13 ± 1.97^{ab}	0.49 ± 0.11^{a}	6.25 ± 0.64^{a}
	t_{20}	13.21 ± 0.79^{a}	0.35 ± 0.11^{a}	4.40 ± 0.14^{bc}

Values are expressed as means ± standard deviations of duplicate independent measurements.
Samples are grouped by batch and displayed according to time. t_0, day of production; t_5, samples after 5 days of ripening; t_{10}, samples after 10 days of ripening; t_{20}, samples after 20 days of ripening.
For each batch different letters in the same column indicate significant differences according to the Tukey–Kramer's (HSD) test (= 0.05).

3.3. Microbiological analyses

The results of viable counts are reported in Table 3.

In more detail, the counts of lactic acid bacteria were comprised between 2.87 and 8.95 log cfu g^{-1}, with statistically lower values recorder at t_0, irrespective of the production batch. No statistically significant differences of lactic acid bacteria counts were highlighted among samples from t_5 to t_{20}.

Regarding coagulase-negative cocci, Enterobacteriaceae, and eumycetes, the counts ranged from 3.45 to 5.94 log cfu g^{-1}, <1 to 2.87 log cfu g^{-1}, and 3.07 to 4.40 log cfu g^{-1}, respectively, with the lowest

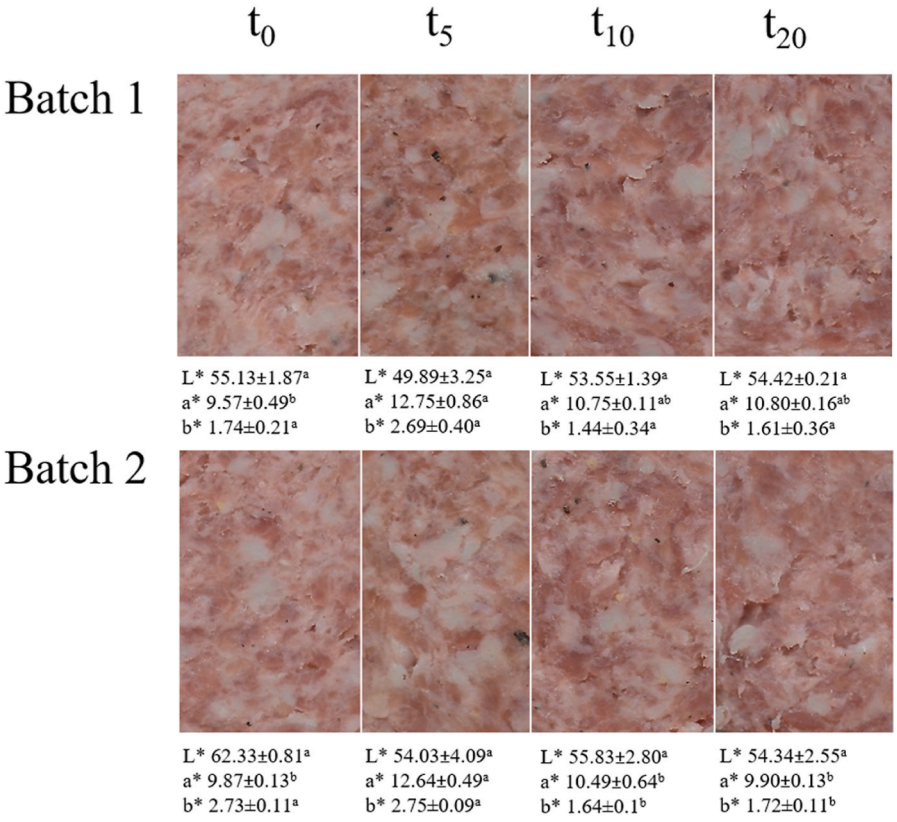


Fig. 2. Images of cross sections of *Ciauscolo* PGI salami during drying
t_0, day of production; t_5, samples after 5 days of ripening; t_{10}, samples after 10 days of ripening; t_{20}, samples after 20 days of ripening, Means ± standard deviations of triplicate independent measurements are shown, Within each batch, for the same color parameter, means followed by different letters are significantly different ($P < 0.05$)., L* value describes the lightness; a* value describes the redness/greenness; b* describes the blueness/yellowness. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Results of viable counting (log cfu g^{-1}) of bacteria and eumycetes in *Ciauscolo* PGI salami during fermentation and ripening.

Batch	Sampling time (days)	Lactic acid bacteria	Coagulase-negative cocci	Enterobacteriaceae	Eumycetes
1	t_0	2.87 ± 0.07^b	3.51 ± 0.01^c	2.87 ± 0.05^a	3.84 ± 0.06^b
	t_5	8.03 ± 0.10^a	4.39 ± 0.14^b	1.99 ± 0.02^b	3.07 ± 0.08^b
	t_{10}	8.13 ± 0.02^a	4.80 ± 0.39^b	1.66 ± 0.23^b	4.40 ± 0.78^a
	t_{20}	8.26 ± 0.07^a	5.94 ± 0.51^a	1.45 ± 0.00^b	3.50 ± 0.22^b
2	t_0	3.42 ± 0.17^b	3.45 ± 0.35^b	1.93 ± 0.18^b	3.10 ± 0.11^b
	t_5	8.58 ± 0.12^a	4.39 ± 0.39^a	<1^c	3.46 ± 0.35^b
	t_{10}	8.47 ± 0.41^a	4.79 ± 0.07^a	<1^c	3.94 ± 0.03^a
	t_{20}	8.95 ± 0.15^a	4.82 ± 0.17^a	<1^c	3.18 ± 0.56^b

Values are expressed as mean ± standard deviation.
Samples are grouped by batch and displayed according to time. t_0, day of production; t_5, samples after 5 days of ripening; t_{10}, samples after 10 days of ripening; t_{20}, samples after 20 days of ripening.
For each batch different letters in the same column indicate significant differences according to the Tukey–Kramer's (HSD) test (= 0.05).

values recorded at t_0, irrespective of the production batch and the microbial group considered.

For *C. botulinum*, the multiplex real-time PCR revealed the absence of the target genes *bont/A, bont/B, bont/E, bont/F,* and 4gyrB *(CP)*, encoding botulinic toxins, in all analyzed samples.

Finally, neither *L. monocytogenes* nor *Salmonella* spp. were detected in 25 g of product, irrespective of the sampling time.

3.4. Metataxonomic analyses

3.4.1. Microbiota

Microbiota of *Ciauscolo* PGI salami significantly differed from t_0 to t_{20} in both the analyzed batches (PERMANOVA, *P*[FDR]<0.05) (Fig. 3). A certain segregation between batch 1 and 2 was anyway observed in the PCoA plot, especially at t_0, although with no statistical significance (*P*[FDR]>0.05). No differences in the alpha diversity parameters were observed between batches and during time (data not shown).

By considering the composition of the microbiota, at t_0, in both batches, three *Pseudomonas* species (*P. fragi, P. psychrophila, P. lundensis*) represented the dominant taxa (Fig. 3). From t_5, *Lat. sakei* was dominant in batch 1 until the end of ripening (t_{20}), whereas in the same timeframe *Dellaglioa algida* represented the most abundant species in batch 2. Unassigned species of *Burkholderia-Caballeronia-Paraburkholderia* and *Escherichia-Shigella* groups were significantly more abundant in the batch 1 (Wilcoxon's Test, *P*[FDR]<0.001). On the other hand, *Leuconostoc carnosum* was detected from t_5 to t_{20}, irrespective of the production batch (Supplementary Table 1).

3.4.2. Mycobiota

As far as the fungi are concerned, a core mycobiota composed by three species of yeast and two genera of mold that cumulatively represented more than 50% of the relative abundance in all samples was observed (Fig. 4, Supplementary Table 2). In fact, *Debaryomyces hansenii*, *Kurtzmaniella zeylanoides* and *Malassezia restricta/globosa* were the most abundant yeasts occurring in all samples, followed by less ubiquitous ASVs. Moreover, molds were mainly represented by *Cladosporium cladosporioides* and *Penicillium* sp., this latter found from t_0 until to t_{10} in batch 2, whereas, in batch 1, it was not observed at t_0 and t_5.

As far as compositional variability of the mycobiota is concerned, PERMANOVA did not highlight any significant effect of time and batch (data not shown).

3.5. Characterization of Lat. sakei isolates

The BLAST search allowed the unambiguous identification of 44 *Lat.*

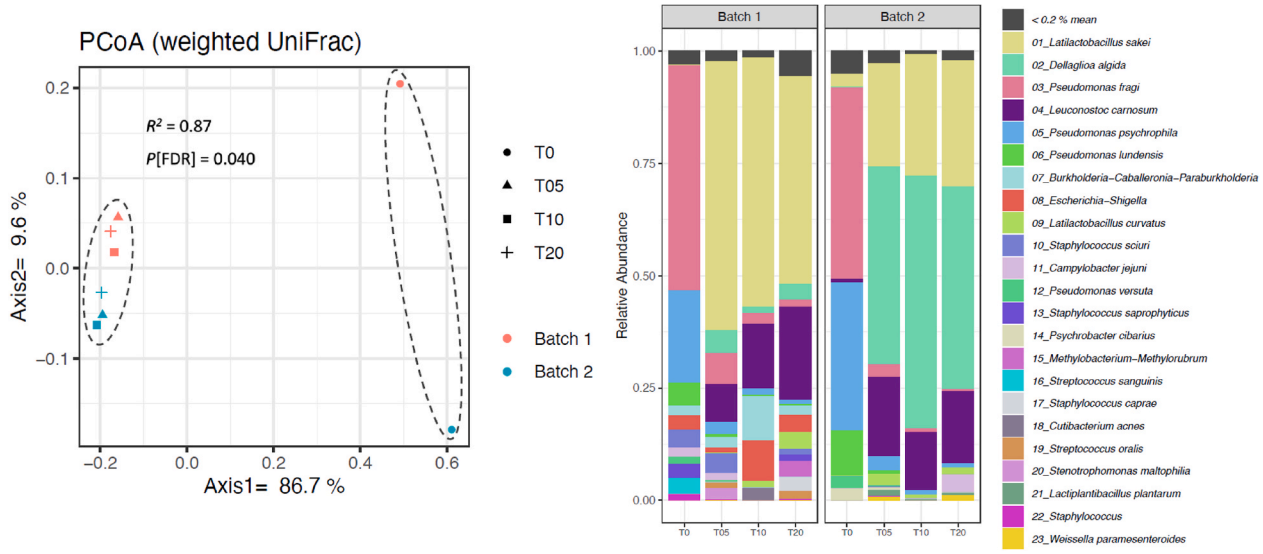


Fig. 3. Microbiota composition
PCoA charts (on the left) displaying weighted UniFrac distance matrix (β-diversity). Batch and sampling time are defined by different colors and shapes (legend); dashed ellipses are indicating significant different communities and results of PERMANOVA are reported in the graph. Stacked bar plots (on the right) showing core microbiota composition (relative abundance) at the species/genus rank level and relative colour coding key. Samples are grouped by batch and displayed according to time; taxa are sorted in the legend from the most to the least abundant (>0.2% of average abundance). t_0, day of production; t_5, samples after 5 days of ripening; t_{10}, samples after 10 days of ripening; t_{20}, samples after 20 days of ripening. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

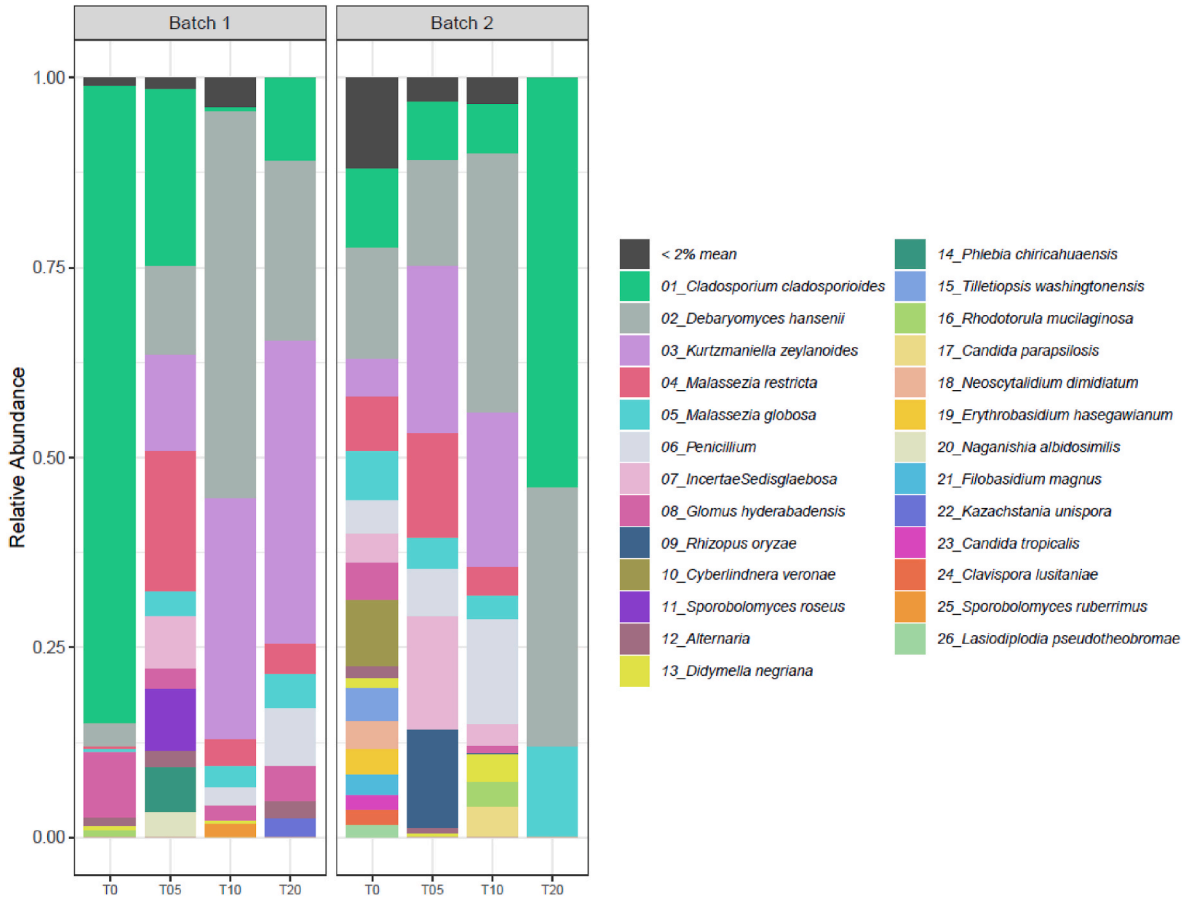


Fig. 4. Mycobiota composition

Stacked bar plots showing core mycobiota composition (relative abundance) at the species/genus rank level and relative colour coding key. Samples are grouped by batch and displayed according to time; taxa are sorted in the legend from the most to the least abundant (>0.2% of average abundance). t_0, day of production; t_5, samples after 5 days of ripening; t_{10}, samples after 10 days of ripening; t_{20}, samples after 20 days of ripening. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

sakei isolates to be obtained; the alignment results of the 16S rRNA sequences obtained from these isolates are reported in Table 4.

Regarding antimicrobial activity assessed through agar well diffusion assay, no *Lat. sakei* isolate showed inhibitory activity against *L. innocua* (Table 4).

As for production of EPS, 21 out of the 44 isolates produced sucrose-dependent EPS, these latter revealed by the formation of mucoid colonies on agar plates (Table 4). By contrast, no isolate showed the production of sucrose-independent EPS.

The result of acidification in the synthetic media assayed are summarized in Fig. 5. In more detail, pH values measured in the uninoculated growth media were 6.30, 6.27, 6.24, 6.11, and 6.13 in MRS, MRS +150 mg kg^{-1} of E250, MRS +150 mg kg^{-1} of E252, MRS +3% NaCl, MRS +150 mg kg^{-1} of E250 + 150 mg kg^{-1} of E252 + 3% NaCl, respectively.

Regarding acidification in MRS broth, according to ANOVA results (data not shown), isolates C55 and C53 showed the highest pH after 24 h (4.91), whereas the isolates C8, C9, C17, C19, C31, C34, C37, and C4 reached the lowest pH values (4.11) after 24 h.

As for acidification in MRS broth added with 150 mg kg^{-1} of E250, according to ANOVA results (data not shown), the isolate *Lat. sakei* C55 showed the highest pH after 24 (4.92), whereas the medium inoculated with the isolates C8, C15, C17, C21, and C29 reached the lowest pH (4.13) after 24 h.

Regarding MRS broth added with 150 mg kg^{-1} of E252, according to ANOVA results (data not shown), again the isolates C55 and C53 showed the highest pH after 24 h, whereas the medium inoculated with the isolate C37 reached the lowest pH value (3.91) after 24 h.

In MRS broth added with 3% NaCl, according to ANOVA results (data not shown), the isolates C50, C51, C53, C54, C55, C57, C59, C60 showed the highest pH values after 24 h (4.71). By contrast, the growth medium inoculated with the isolates C17, C29, C31, or C37 reached the lowest pH (3.93) after 24 h.

As for MRS broth added with 150 mg kg^{-1} of E250 + 150 mg kg^{-1} of E252 + 3% NaCl, according to ANOVA results (data not shown), the isolate C2 showed the lowest pH drop after 24 h (5.02), whereas the medium inoculated with the isolate C8 reached the lowest pH (3.92) after 24 h.

For all these growth media, according to ANOVA, a wide variability of the results was observed among the isolates (data not shown).

The results of the semi-quantitative assessment of selected enzymatic activities are reported in the heat map represented in Fig. 6.

According to the manufacturer's instructions, only the isolates with color development, scored as 3, 4, or 5, were considered as positive for the tested enzymatic activity.

As for alkaline phosphatase, only two isolates (C4 and C15) showed a positive reaction. Thirty-nine out of the 44 *Lat. sakei* isolates showed a strong activity for leucine arylamidase, whereas 36 isolates showed a positive reaction for valine arylamidase. Acid phosphatase activity was observed in 27 isolates, whereas only 10 isolates were positive for naphthol-AS-BI-phosphohydrolase activity. Six isolates were positive for alpha-galactosidase, whereas 18 showed a positive reaction for beta-galactosidase. Alpha-glucosidase, beta-glucosidase, and N-acetyl-ß-glucosaminidase were observed in 3 isolates, being C16, C28, and C39.

Table 4
Identification and characterization of lactic acid bacteria isolates from *Ciauscolo* PGI salami.

Production batch	Isolate	GenBank Accession no.	Species (Accession no.)	% identity	Antimicrobial activity	EPS production		*hdcA* gene
						Sucrose-dependent	Sucrose-independent	
1	C1	ON303755	*Latilactobacillus sakei* (NR_113821)	99.3%	–	–	–	–
	C2	ON303756	*Latilactobacillus sakei* (NR_113821)	99.5%	–	–	–	–
	C3	ON303757	*Latilactobacillus sakei* (NR_113821)	99.6%	–	–	–	–
	C4	ON303758	*Latilactobacillus sakei* (NR_113821)	98.7%	–	–	–	–
	C5	ON303759	*Latilactobacillus sakei* (NR_113821)	99.6%	–	M	–	–
	C6	ON303760	*Latilactobacillus sakei* (NR_113821)	99.9%	–	–	–	–
	C7	ON303761	*Latilactobacillus sakei* (NR_113821)	99.5%	–	M	–	–
	C8	ON303762	*Latilactobacillus sakei* (NR_113821)	99.4%	–	–	–	–
	C9	ON303763	*Latilactobacillus sakei* (NR_113821)	99.8%	–	–	–	–
	C10	ON303764	*Latilactobacillus sakei* (NR_113821)	99.9%	–	–	–	–
	C11	ON303765	*Latilactobacillus sakei* (NR_113821)	99.0%	–	M	–	–
	C12	ON303766	*Latilactobacillus sakei* (NR_113821)	99.7%	–	–	–	–
	C13	ON303767	*Latilactobacillus sakei* (NR_113821)	99.5%	–	–	–	–
	C14	ON303768	*Latilactobacillus sakei* (NR_113821)	99.1%	–	–	–	–
	C15	ON303769	*Latilactobacillus sakei* (NR_113821)	99.6%	–	–	–	–
	C17	ON303785	*Latilactobacillus sakei* (NR_113821)	98.7%	–	M	–	–
	C18	ON303787	*Latilactobacillus sakei* (NR_113821)	99.5%	–	M	–	–
	C19	ON303788	*Latilactobacillus sakei* (NR_113821)	98.9%	–	M	–	–
	C21	ON303791	*Latilactobacillus sakei* (NR_113821)	99.2%	–	M	–	–
	C24	ON303794	*Latilactobacillus sakei* (NR_115172)	99.5%	–	M	–	–
	C26	ON303796	*Latilactobacillus sakei* (NR_113821)	99.5%	–	M	–	–
	C27	ON303799	*Latilactobacillus sakei* (NR_113821)	99.7%	–	M	–	–
2	C29	ON303771	*Latilactobacillus sakei* (NR_113821)	99.5%	–	M	–	–
	C31	ON303772	*Latilactobacillus sakei* (NR_113821)	98.9%	–	M	–	–
	C33	ON303773	*Latilactobacillus sakei* (NR_113821)	98.8%	–	M	–	–
	C34	ON303775	*Latilactobacillus sakei* (NR_113821)	99.9%	–	M	–	–
	C37	ON303778	*Latilactobacillus sakei* (NR_113821)	99.6%	–	M	–	–
	C40	ON303780	*Latilactobacillus sakei* (NR_113821)	99.9%	–	M	–	–
	C42	ON303781	*Latilactobacillus sakei* (NR_113821)	99.0%	–	–	–	–
	C45	ON303783	*Latilactobacillus sakei* (NR_113821)	99.5%	–	M	–	–
	C46	ON303800	*Latilactobacillus sakei* (NR_113821)	99.9%	–	–	–	–
	C47	ON303801	*Latilactobacillus sakei* (NR_113821)	98.6%	–	–	–	–
	C48	ON303802	*Latilactobacillus sakei* (NR_113821)	99.0%	–	M	–	–
	C49	ON303803	*Latilactobacillus sakei* (NR_113821)	99.3%	–	–	–	–
	C50	ON303804	*Latilactobacillus sakei* (NR_113821)	98.8%	–	–	–	–
	C51	ON303805	*Latilactobacillus sakei* (NR_113821)	98.8%	–	–	–	–

(*continued on next page*)

Table 4 (*continued*)

Production batch	Isolate	GenBank Accession no.	Species (Accession no.)	% identity	Antimicrobial activity	EPS production		*hdcA* gene
						Sucrose-dependent	Sucrose-independent	
	C53	ON303807	*Latilactobacillus sakei* (NR_113821)	99.8%	–	M	–	–
	C54	ON303808	*Latilactobacillus sakei* (NR_113821)	99.3%	–	–	–	–
	C55	ON303809	*Latilactobacillus sakei* (NR_113821)	99.1%	–	M	–	–
	C56	ON303810	*Latilactobacillus sakei* (NR_113821)	99.2%	–	–	–	–
	C57	ON303811	*Latilactobacillus sakei* (NR_113821)	100%	–	–	–	–
	C58	ON303812	*Latilactobacillus sakei* (NR_113821)	99.0%	–	–	–	–
	C59	ON303813	*Latilactobacillus sakei* (NR_113821)	98.6%	–	–	–	–
	C60	ON303814	*Latilactobacillus sakei* (NR_113821)	99.9%	–	M	–	–

-, negative; M, mucoid colonies.

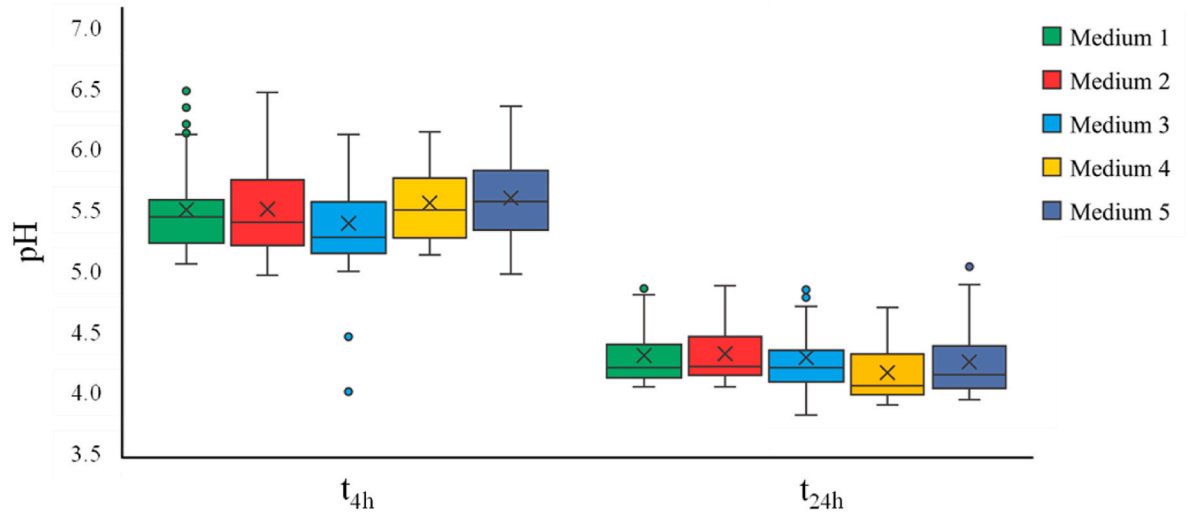


Fig. 5. Box plots summarizing the results of acidification activity of the *Latilactobacillus sakei* isolates in synthetic media after 4 and 24h. For each box, the bottom whisker marks the minimum value, the bottom of the box marks the location of first quartile, the line within the box refers to the median value, the top of the box marks the location of the third quartile, the top whisker marks the maximum value in the data set, the "X" symbol marks the average value, and circles indicate the outliers
Medium 1, MRS broth
Medium 2, MRS broth +150 mg kg^{-1} E250
Medium 3, MRS broth +150 mg kg^{-1} E252
Medium 4, MRS broth +3% NaCl
Medium 5, MRS broth +150 mg kg^{-1} E250 + 150 mg kg^{-1} E252 + 3% NaCl.

Finally, no isolate was positive for esterase (C 4), esterase lipase (C 8), lipase (C 14), cystine arilamidase, trypsin, alpha-chymotripsin, beta-glucuronidase, alpha-mannosidase, or alpha-fucosidase activity.

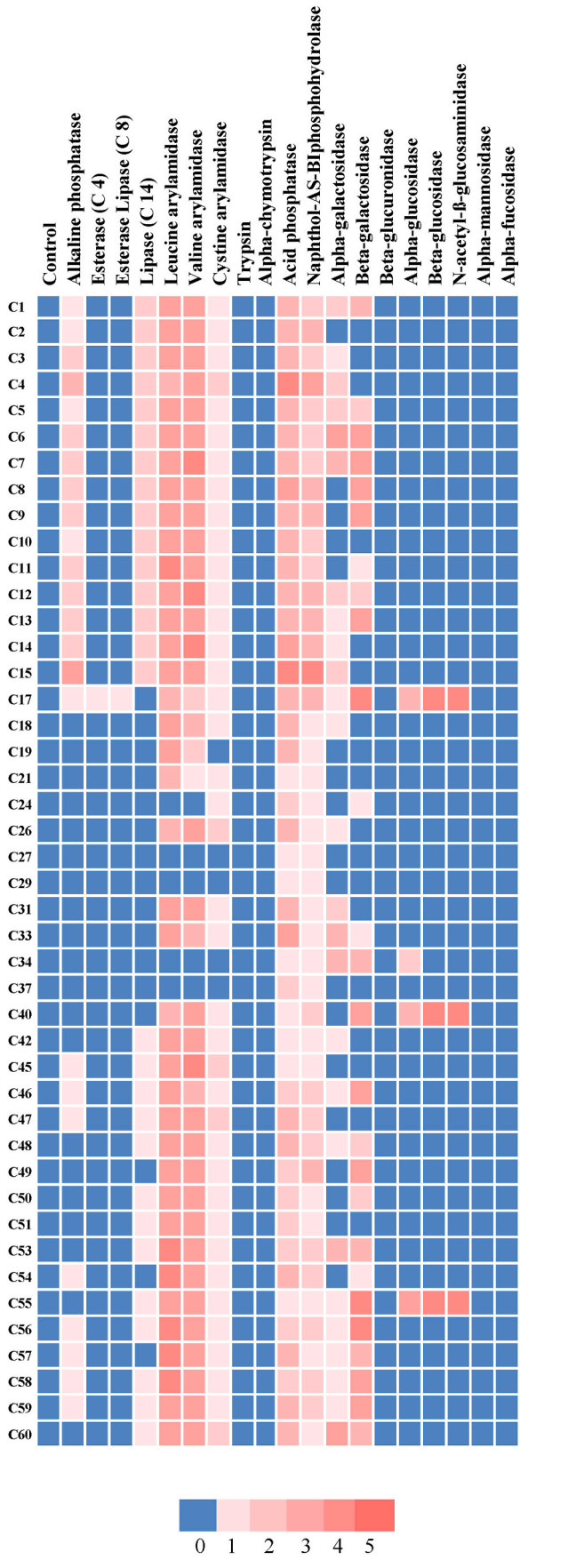
Regarding the quantification of the *hdcA* gene, no positive *Lat. sakei* isolate was observed (Table 4).

3.6. GC-MS analysis of volatile components

Volatile compounds detected in the static headspace of the two batches of *Ciauscolo* PGI salami samples are reported in Table 5.

Monoterpene hydrocarbons (limonene, sabinene, α-pinene, β-pinene, 3-carene, α-thujene) and sesquiterpene hydrocarbons (β- and α-copaene) dominated the aroma profiles of samples, throughout the whole ripening time. Allyl methyl sulphide and diallyl disulphide were the major aliphatic sulphur compounds, but lower amounts of diallyl sulphide and allyl methyl disulphide were also detected. Samples from batch 1 were characterized by higher levels of monoterpene hydrocarbons and alcohols (n-hexanol and n-pentanol, mainly) and lower levels of sulphur compounds than those belonging to batch 2. Different dynamics were observed in the aroma composition during the ripening. In more detail, increasing levels of monoterpene hydrocarbons (sabinene, α-pinene, camphene) and 2-methyl-1-butanol, and decreasing amounts of 1-pentanol, ethyl hexanoate, and octanal were observed in samples from batch 1; whereas samples from batch 2 were characterized by very limited changes in the aroma composition during ripening, where only an increase of ethyl ester levels (ethyl isopentanoate) and a decrease of 1-pentanol were observed.

Regarding the correlation between bacteria-fungi and VOCs, positive and negative correlations (*P*-value [FDR adjusted] <0.05) mainly influenced by the batch and then by the sampling time were observed



(caption on next column)

Fig. 6. Heat map representing the results of semi-quantitative assessment of enzymatic activities of *Latilactobacillus sakei* (C1–C60) isolated from *Ciauscolo* PGI salami.
For each enzymatic reaction, a value ranging from 0 to 5 was assigned, corresponding to the colors developed: 0 corresponds to a negative reaction (dark blue dots), 5 to a reaction of maximum intensity (dark red dots), and values 1, 2, 3 or 4 are intermediate reactions depending on the level of intensity (3, 4, or 5 being considered as positive reactions). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 7). *Lat. sakei* was negatively correlated with *cis*-sabinene hydrate and positively correlated with ethyl isopentanoate (ester) and with 3-methyl-1-butanol, together with *Latilactobacillus curvatus*, whereas *D. algida* was positively correlated with allyl-methyl disulfide and α-thujene. *Lactiplantibacillus plantarum* showed positive relationship with ethyl pentanoate and 1-pentanol. Regarding fungi, a direct relationship of *D. hansenii* and *K. zeylanoides* with β-caryophillene and 2-methyl-1-butanol (alcohol), was respectively observed.

4. Discussion

The microbiota of fermented sausages has widely been investigated along time; however, traditional fermented sausages still represent a niche of undisclosed microbial diversity and a source of pro-technological microorganisms that could successfully be applied by the industry of fermented meat for product improvement. In the present study, the morpho-textural, physico-chemical (including volatile compounds), and microbiological characteristics of *Ciauscolo* PGI salami during ripening were investigated. *Lat. sakei* isolates collected from the analyzed samples were also tested for some pro-technological activities to select potential cultures to be used as starter or adjuncts.

As for pH values detected in the analyzed samples, the data were in accordance with those already detected by Belleggia et al. (2020a) and Trani et al. (2010) in *Ciauscolo* salami produced by other manufacturers. In the analyzed samples, a progressive decrease in pH was observed during time, irrespective of the production batch. Of note, since *Ciauscolo* is ready for consumption already after 20 days, the pH values herein detected in the end products reflect the short ripening time. According to the production disciplinary, the pH of ready-to-eat *Ciauscolo* PGI salami should be $\geq$ 4.8. In accordance with pH reduction, an increase in TTA values as well as in lactic and acetic acid content was also observed in the analyzed samples. The trends of the abovementioned parameters can likely be explained by the activity of the lactic acid bacteria naturally occurring in the raw material which produced organic acids through fermentative metabolism of meat carbohydrates (Fadda et al., 2010). Of note, the presence of both lactic and acetic acid in the analyzed samples attests the presence of homofermentative as well as heterofermentative lactic acid bacteria. The presence of acetic acid could also be the result of the metabolic activity of coagulase-negative staphylococci as observed by Li et al. (2023a).

In the analyzed samples, a reduction of a_w was observed during time, thus attesting the progressive drying of the product with further improvement of the safety and stability of *Ciauscolo* PGI salami. The detected values were in accordance with those reported by Trani et al. (2010) and Belleggia et al. (2020a) in the same type of fermented sausage.

Interestingly, pH and a_w values herein detected were higher than those detected by Aquilanti et al. (2007) in the same type of fermented sausage, thus suggesting a great variation of these parameters based on the artisan method applied during production.

Based on Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, ready-to-eat products with pH > 4.4 or a_w > 0.92 or products with pH > 5.0 and a_w > 0.94 are prone to support the growth of *L. monocytogenes*, hence, such issue should be taken into consideration when producing *Ciauscolo* PGI salami, whose pH and a_w

Table 5
Volatile compounds (mean[a] ± SD; n = 3) detected in *Ciauscolo* PGI salami headspaces during fermentation and ripening.

RI	Name	Class	Batch 1				Batch 2			
			t_0	t_5	t_{10}	t_{20}	t_0	t_5	t_{10}	t_{20}
701	allyl methyl sulfide	SU	49 ± 4	29 ± 28	6 ± 2	23 ± 3	66 ± 7	64 ± 7	57 ± 2	72 ± 6
735	3-methyl-1-butanol	AL	16 ± 3	43 ± 34	62 ± 8	59 ± 3	7 ± 2	15 ± 6	19 ± 5	20 ± 7
742	2-methyl-1-butanol	AL	8 ± 1^{b}	10 ± 1^{b}	31 ± 4^{a}	30 ±2^{a}	3 ± 1	8 ± 3	10 ± 3	8 ± 2
768	1-pentanol	AL	129 ± 17^{a}	91 ± 5^{ab}	63 ± 1^{b}	69 ± 10^{b}	89 ± 25^{a}	28 ± 7^{b}	31 ± 5^{b}	20 ± 1^{b}
804	hexanal	AD	1103 ± 119	516 ± 481	113 ± 19	136 ± 39	744 ± 347	80 ± 55	185 ± 86	161 ± 53
859	ethyl isopentanoate	ES	2 ± 0	7 ± 4	11 ± 1	8 ± 1	0 ± 0^{b}	1 ± 0^{b}	5 ± 1^{a}	4 ± 1^{a}
865	diallyl sulfide	SU	2 ± 0	3 ± 1	2 ± 0	2 ± 1	7 ± 1	10 ± 9	10 ± 8	5 ± 1
874	hexanol	AL	79 ± 16	118 ± 2	277 ± 10	372 ± 156	21 ± 11	36 ± 27	59 ± 3	27 ± 3
902	heptanal	AD	38 ± 4	29 ± 18	11 ± 1	13 ± 2	18 ± 14	6 ± 5	16 ± 5	12 ± 3
921	allyl methyl disulfide	SU	3 ± 1	9 ± 4	3 ± 1	4 ± 1	2 ± 0	9 ± 6	9 ± 6	7 ± 0
933	α-thujene	MH	494 ± 42	507 ± 17	516 ± 8	590 ± 11	374 ± 81	376 ± 127	504 ± 34	491 ± 6
941	α-pinene	MH	643 ± 34^{b}	698 ± 45^{ab}	715 ± 2^{ab}	823 ± 38^{a}	265 ± 24	321 ± 119	386 ± 10	388 ± 8
956	camphene	MH	25 ± 0^{b}	26 ± 2^{ab}	25 ± 1^{ab}	30 ± 0^{a}	11 ± 1	12 ± 4	15 ± 1	15 ± 0
975	heptanol	AL	8 ± 4	6 ± 4	14 ± 1	16 ± 3	3 ± 0	3 ± 2	4 ± 0	2 ± 1
979	sabinene	MH	662 ± 24^{b}	596 ± 109^{b}	944 ± 47^{a}	922 ± 41^{a}	510 ± 22	651 ± 352	813 ± 44	735 ± 51
982	β-pinene	MH	1132 ± 23	944 ± 291	1309 ± 35	1436 ± 127	490 ± 10	517 ± 175	629 ± 61	593 ± 20
986	2,3-octanedione	KE	20 ± 3	12 ± 11	1 ± 1	17 ± 6	84 ± 82	6 ± 3	9 ± 3	9 ± 2
993	myrcene	MH	291 ± 12	270 ± 35	327 ± 11	345 ± 39	169 ± 11	169 ± 68	220 ± 14	200 ± 11
999	ethyl hexanoate	ES	31 ± 0^{ab}	47 ± 12^{a}	23 ± 3^{ab}	12 ± 0^{b}	1 ± 0	5 ± 5	15 ± 4	9 ± 0
1003	octanal	AD	14 ± 2^{a}	15 ± 2^{a}	4 ± 1^{b}	6 ± 0^{b}	10 ± 9	3 ± 1	5 ± 2	3 ± 0
1007	α-phellandrene	MH	31 ± 1	31 ± 6	25 ± 3	31 ± 3	81 ± 6	74 ± 22	68 ± 1	67 ± 1
1014	3-carene	MH	219 ± 15	259 ± 21	285 ± 22	269 ± 21	370 ± 2	449 ± 168	520 ± 66	482 ± 34
1021	α-terpinene	MH	131 ± 15	135 ± 5	119 ± 6	147 ± 7	121 ± 28	110 ± 36	147 ± 20	140 ± 7
1029	p-cymene	MH	87 ± 6	74 ± 1	80 ± 1	85 ± 3	64 ± 9	78 ± 35	97 ± 0	84 ± 3
1035	limonene	MH	1986 ± 31	2038 ± 213	2312 ± 110	2413 ± 310	1158 ± 54	1221 ± 453	1455 ± 46	1363 ± 45
1043	(Z)-β-ocimene	MH	4 ± 1	4 ± 0	4 ± 0	5 ± 2	12 ± 0	11 ± 4	12 ± 1	12 ± 0
1064	γ-terpinene	MH	253 ± 13	244 ± 15	215 ± 9	262 ± 13	216 ± 54	189 ± 61	254 ± 27	246 ± 15
1073	*cis*-sabinene hydrate	OM	19 ± 1	10 ± 5	11 ± 6	6 ± 1	10 ± 5	8 ± 3	9 ± 0	8 ± 2
1083	diallyl disulfide	SU	11 ± 3	21 ± 13	13 ± 8	18 ± 11	26 ± 0	42 ± 36	58 ± 49	23 ± 2
1091	terpinolene	MH	95 ± 11	99 ± 14	88 ± 3	115 ± 3	83 ± 10	74 ± 21	118 ± 7	111 ± 1
1184	1-terpinen-4-ol	OM	8 ± 0	10 ± 1	9 ± 1	9 ± 1	12 ± 5	9 ± 3	12 ± 1	10 ± 1
1198	ethyl octanoate	ES	5 ± 2	5 ± 3	10 ± 9	3 ± 2	2 ± 0	0 ± 0	2 ± 1	2 ± 1
1345	δ-elemene	SH	6 ± 0	8 ± 1	11 ± 1	13 ± 3	13 ± 1	12 ± 6	15 ± 1	11 ± 1
1357	α-cubebene	SH	7 ± 0	8 ± 1	10 ± 1	54 ± 62	2 ± 0	2 ± 1	3 ± 0	2 ± 0
1375	ylangene	SH	10 ± 12	2 ± 0	3 ± 0	3 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
1384	α-copaene	SH	60 ± 4^{b}	67 ± 6^{ab}	84 ± 12^{ab}	39 ± 6^{a}	18 ± 4	17 ± 8	22 ± 0	17 ± 0
1398	β-elemene	SH	3 ± 0^{c}	4 ± 0^{bc}	6 ± 1^{ab}	7 ± 1^{a}	4 ± 1	4 ± 2	6 ± 2	4 ± 0
1416	β-caryophillene	SH	1 ± 0^{b}	2 ± 2^{b}	17 ± 6^{a}	11 ± 0^{ab}	2 ± 0	4 ± 0	7 ± 4	5 ± 1
1418	α-gurjunene	SH	1 ± 0	1 ± 0	2 ± 0	2 ± 0	0 ± 0	4 ± 2	4 ± 2	4 ± 1
1430	β-copaene	SH	222 ± 22	257 ± 38	323 ± 45	377 ± 67	316 ± 51	304 ± 151	398 ± 33	312 ± 16
1438	germacrene D	SH	3 ± 0	3 ± 0	3 ± 0	4 ± 1	3 ± 1	3 ± 1	3 ± 0	3 ± 0
1460	allo-aromadendrene	SH	2 ± 0	2 ± 0	2 ± 0	3 ± 1	3 ± 0	2 ± 1	3 ± 0	2 ± 0
1464	α-caryophyllene	SH	8 ± 1	10 ± 1	13 ± 3	15 ± 4	12 ± 1	12 ± 6	17 ± 2	12 ± 1
1482	β-cadinene	SH	1 ± 0	1 ± 0	1 ± 0	1 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1485	γ-muurolene	SH	1 ± 0	1 ± 0	1 ± 0	1 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1496	β-guaiene	SH	1 ± 0	2 ± 0	3 ± 1	4 ± 3	2 ± 0	2 ± 1	6 ± 3	5 ± 4
1504	α-selinene	SH	1 ± 0	1 ± 0	2 ± 1	2 ± 1	2 ± 1	1 ± 1	3 ± 0	2 ± 0
1507	α-muurolene	SH	1 ± 0	1 ± 0	1 ± 0	2 ± 0	1 ± 0	1 ± 0	2 ± 1	1 ± 0
1527	δ-cadinene	SH	4 ± 0	5 ± 1	6 ± 1	6 ± 1	2 ± 0	2 ± 1	2 ± 0	2 ± 0
1536	cadina-1,4-diene	SH	1 ± 0	1 ± 0	1 ± 0	2 ± 0	1 ± 0	0 ± 0	1 ± 0	1 ± 0

Means with different superscripts in the same row and for the same batch are different at probability level of 0.95.
RI, experimental Kovats Retention Index calculated for a DB-5 type capillary column. SU, sulphur compound. AL, alcohol. AD, aldehyde. ES, ester. MH, monoterpene hydrocarbon. KE, ketone. OM, oxygenated monoterpene. SH, sesquiterpene hydrocarbon.
Samples are grouped by batch and displayed according to ripening time (t_0, t_5, t_{10}, t_{20} days of ripening).
[a] Chromatographic peak areas (arbitrary units × 10^4).

values can sometimes be permissive for the growth of the pathogen.

For color measurements, it is noteworthy that *Ciauscolo* PGI salami is a short-time ripened fermented sausage, hence, the color of the end product (at t_{20}) may not substantially differ from that of the stuffed meat batter at t_0. To the authors knowledge, no data reporting the color of *Ciauscolo* PGI salami are available in the scientific literature for further comparison of results. Regarding lightness, this parameter can vary from 0 (black) to 100 (white) (Méndez-Cid et al., 2017); hence, the high level of lightness detected in the samples herein analyzed can likely be attributed to the high amount of fat that characterizes the meat batter of this fermented sausage (Estévez et al., 2005). As for a* parameter, this axis represents the green–red opponent colors, with values < 0 toward green and values > 0 toward red (Méndez-Cid et al., 2017). In fermented sausages, the intensity of the red color is enhanced by the presence of nitrates and nitrites as well as by the microbial activity of pro-technological microorganisms (e.g., lactic acid bacteria and coagulase-negative cocci) (Holck et al., 2017). In the samples herein analyzed, the a* values were in the range of the red color, with slight variations detected only at t_5. Regarding the b* parameter, this axis denotes the blue–yellow opponents, with values < 0 toward blue and those >0 toward yellow. In the present study, samples showed values of yellow around 1–2, with only slight variations occurring at t_{10} and t_{20} in the samples of batch 2. In general, an increase of b* value in fermented sausages could denote fat rancidity due to lipid oxidation (Méndez-Cid et al., 2017), hence, the low values detected in the samples herein analyzed represent a positive quality characteristic of this high-containing lard salami and suggest a low level of lipid oxidation.

Regarding texture, as expected, the longer the ripening time, the

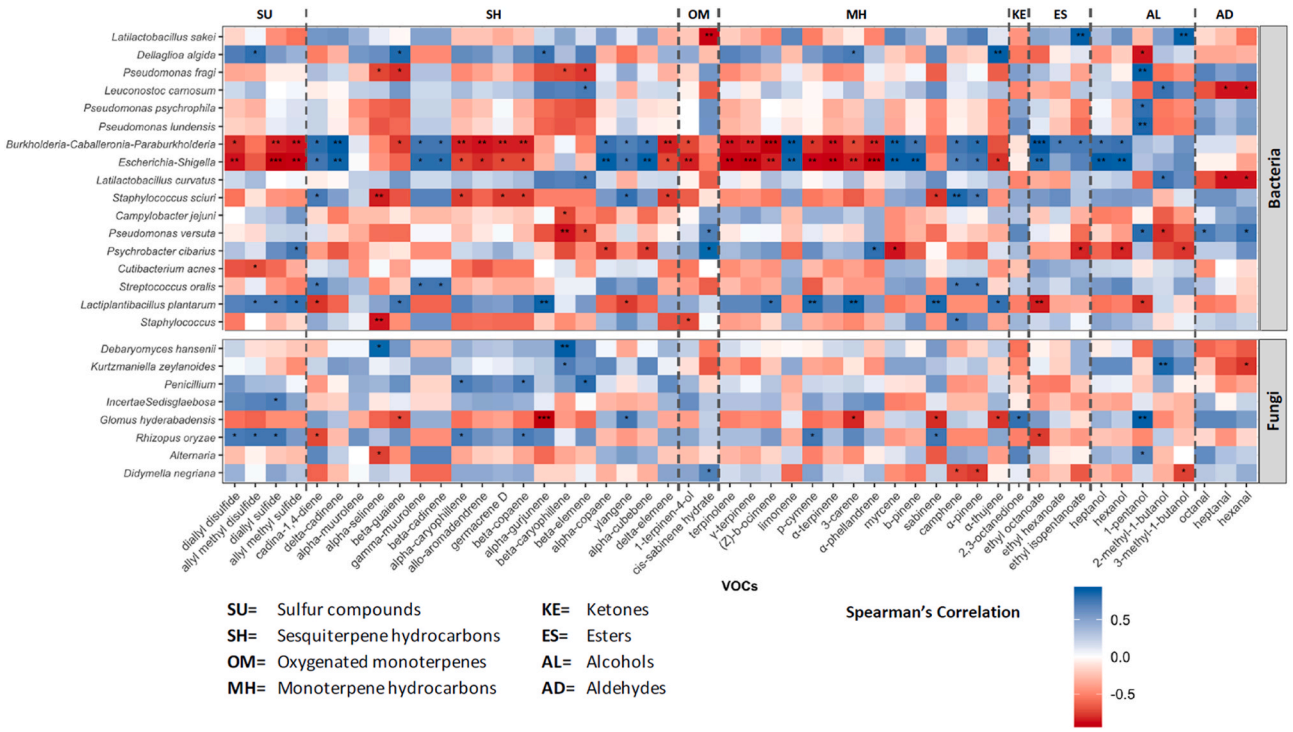


Fig. 7. Correlation between metataxonomic and volatilome data
Tile plots showing the existing pairwise correlations between VOCs and bacterial-fungal ASVs (merged at the species/genus level). Taxa are ordered from the most to the least abundant, whereas the VOCs are grouped in relation to their chemical class (refer to the caption for the codes). Colors represent level of Spearman's Rho correlation (from −1 to 1; caption) and significant positive and negative correlations are highlighted with asterisks (*P*-value [FDR adjusted]: *= <0.05; **= <0.01; ***= <0.001). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

higher the measured hardness. This evidence was also supported by the lower a_w values measured in the samples at t_{20} in respect with those at t_0, thus highlighting the achievement of the required drying of the product. As for the cohesiveness, in fermented sausages, this parameter is affected by the solubility of muscle proteins (Farouk et al., 2002); moreover, as reported by Bis-Souza et al. (2020), the amount of fat strongly influences this parameter. In more detail, Bis-Souza et al. (2020) observed a progressive increase of cohesiveness as well as of springiness in salami produced with a progressive reduction of pork fat. Interestingly, the values of cohesiveness measured in the samples herein analyzed were as low as those reported by Bis-Souza et al. (2020) for Italian salami containing 20% (w w^{-1}) of pork fat, attesting at about 0.5.

Viable counts carried out on *Ciauscolo* PGI salami samples highlighted a progressive increase of pro-technological microbial groups as well as a decrease of indicators of enteric contamination.

As it has previously been elucidated, raw meat can be contaminated by pathogenic or spoilage microorganisms during animal slaughtering (Taylor and Aiyegoro, 2022). In more detail, the Enterobacteriaceae family includes important foodborne pathogens such as *Salmonella* spp., *Yersinia enterocolitica*, *Escherichia coli* (including the pathogenic species *E. coli* O157:H7), *Shigella* spp., and *Cronobacter* spp. Enterobacteriaceae, together with coliforms, are often assessed in foods to highlight poor hygiene or inadequate processing, process failure, or post-process contamination, being therefore considered as indicator microorganisms (Petruzzelli et al., 2016). In the present study, the two analyzed batches were characterized by a constant reduction in Enterobacteriaceae counts that corresponded to a notable increase in pro-technological microorganisms as lactic acid bacteria and coagulase-negative cocci. Of note, for Enterobacteriaceae, pH tolerance is often influenced by the acidulant they are exposed to, with lactic acid being more inhibitory than mineral acids. This feature explains the progressive reduction of Enterobacteriaceae in the analyzed samples at t_{20}, where the amount of lactic acid was maximum.

Lactic acid bacteria represent a key microbial group in meat fermentation. Their metabolic activity improves the safety and sensory quality of fermented sausages (Fadda et al., 2010). Indeed, lactic acid and other organic acids (e.g., acetic acid) produced by lactic acid bacteria prevent the development of spoilage and pathogenic microorganisms. Of note, some strains of lactic acid bacteria can act as protective cultures in fermented meat products through the production of bacteriocins. These compounds are proteins with potential antimicrobial activity against specific pathogens (e.g., *L. monocytogenes*) (Junges da Costa et al., 2021). Moreover, lactic acid bacteria metabolism leads to the stabilization of meat color and to texture development (e.g., formation of a distinctive gel-like texture due to protein denaturation) (Fadda et al., 2010). Lactic acid bacteria are also responsible for the production of volatile compounds through the release of free amino acids (Fadda et al., 2010). In the present study, the counts of lactic acid bacteria were consistent with those already detected by Belleggia et al. (2020a) and Aquilanti et al. (2007) in the same salami at 20 days of ripening, attesting at about 8 log cfu g^{-1}.

In fermented meat, coagulase-negative cocci enhance the aroma and texture of the product. In more detail, this microbial group encompasses microorganisms that produce nitrate reductase, this latter enzyme converting nitrate to nitrite (Khusro and Aarti, 2022). Nitrites exert an antimicrobial activity against clostridia, especially *C. botulinum*, with a consequent reduction of food poisoning risk; they also exert an antioxidative activity and promote the formation of nitrosomyoglobin with the subsequent development and stabilization of a pleasant red color (Khusro and Aarti, 2022). Furthermore, coagulase-negative cocci improve flavor and aroma of fermented sausages through the formation of volatile compounds deriving from proteolysis and lipolysis (Khusro and Aarti, 2022). In the present study, an increase in coagulase-negative cocci counts was observed during time, with final counts that were similar to those already detected by Aquilanti et al. (2007) in *Ciauscolo* PGI at 20 days of ripening, but higher than those recently detected by

Belleggia et al. (2020a) in the same salami, thus suggesting the occurrence of a certain variability among producers based on production process and the raw materials used.

As for eumycetes (yeasts and molds), their role in fermented sausages is mainly related to the production of proteolytic (exoproteases) and lipolytic (exolipases) enzymes that are responsible for the development of flavor and aroma (Copetti, 2019; Sunesen and Stahnke, 2003). Of note, yeasts are common environmental contaminants of fermented sausages and are well adapted to this high-salt and acidic environment (Osei Abunyewa et al., 2000). As reported by Cocolin et al. (2011), molds occurring on the surface of fermented sausages facilitate the dehydration process, since they create micro-pores on the casing. Finally, external mold layer protects lipids from oxidation in the presence of light (Cocolin et al., 2011).

Of note, the absence of the major pathogens as *L. monocytogenes*, *Salmonella* spp., and of the target genes *bont/A, bont/B, bont/E, bont/F,* and 4gyrB *(CP)*, encoding for botulinic toxins, in all the samples herein analyzed attests the high quality of the raw materials and the proper application of good manufacturing practices.

Metataxonomic analysis highlighted differences in the microbiota of the analyzed samples, including stuffed meat batter, fermented sausages sampled during ripening, and fermented sausages sampled at the end of maturation; in more detail, a progressive substitution of meat spoilage bacteria of with pro-technological microorganisms was seen.

Pseudomonas, that encompasses psychotropic bacteria, constituted the core microbiota of stuffed salami at t_0. *P. fragi* is a spoilage microorganism that produces slime on meat during storage and it is responsible for off-odors and soften of the flesh (Shao et al., 2021). *P. psychrophila* is a facultatively psychrophilic bacterium that can be responsible for meat spoilage (Yumoto et al., 2001). Regarding *P. lundensis*, this is a further spoilage species that has already been isolated from beef and lamb fresh meat, where it was responsible for the production of off-odors (Shao et al., 2021). As reported by Fidan et al. (2022), the presence of organic acids (as lactic or acetic acid) can inhibit putrefactive bacteria, including those belonging to the genus *Pseudomonas*, thus explaining the reduction of such microorganisms as the fermentation of the sausages herein analyzed has progressed.

As for the most represented pro-technological species, *Lat. sakei* and *Leuc. carnosum* dominated in salami of batch 1 after 5 days of ripening, whereas the same two species, together with *D. algida*, were mostly represented in samples of batch 2.

Lat. sakei represents the emblematic lactic acid bacteria species in fermented meat products for its rapid acid production (Amadoro et al., 2015; Zagorec and Champomier-Vergès, 2017). Of note, *Lat. sakei* also represented the sole lactic acid bacteria species isolated from the analyzed samples, thus confirming the assertiveness of this species in sausage fermentation (Janßen et al., 2020).This psychrotrophic and facultative anaerobic species has already been recognized as the key lactic acid bacterium in fermented sausages produced in Western Europe, as well as in fermented meat products manufactured in Asia and South America (Zagorec and Champomier-Vergès, 2017). In meat or fermented sausages, *Lat. sakei* takes advantage of meat nutrients by encoding oligopeptide transporters and intracellular peptidases through genes up-regulation. The endo and exo-peptidases produced by *Lat. sakei* increase the concentration of free amino acids, thus positively affecting flavor development (Belleggia et al., 2022b). Moreover, *Lat. sakei* can use the ribose present in raw meat as carbon source through an ATP-dependent system (Zagorec and Champomier-Vergès, 2017). Based on the abovementioned features, in fermented sausages, *Lat. sakei* shows a shorter lag phase and a growth rate higher than other lactic acid bacteria; moreover, it is highly salt tolerant (up to 6.5% NaCl) (Amadoro et al., 2015).

As reviewed by Honrada Perez et al. (2022), *Lat. sakei* can produce multiple bacteriocins, namely sakacin P, sakacin T, and sakacin X, depending on the strain. At this regard, none of the 44 *Lat. sakei* cultures herein isolated showed antimicrobial activity against *L. innocua*. The production of bacteriocins represents a highly desirable feature due to the competitive advantage obtained by the producing strain and for the increased safety of the product. Of note, the genes encoding for active bacteriocins are frequently located in operon clusters, harbored in the genome, plasmid, or in other mobile genetic elements; moreover, the expression of these operons is complex and can be induced by the presence of auto-inducer peptides (Kumariya et al., 2019). The abovementioned features might explain the lack of bacteriocins production in the analyzed *Lat. sakei* isolates and suggest that bacteriocin production by *Lat. sakei* is less common than expected as already reported by Belleggia et al. (2022c), who observed no listericidal effect of 22 *Lat. sakei* cultures isolated from fermented fish sausages.

As reported by Wang et al. (2019), strains of *Lat. sakei* also showed a high-yield EPS production with unique characteristics for their exploitation in large-scale industrial food applications. EPS produced in fermented sausages by lactic acid bacteria can impact on the morpho-textural properties of the end product (Hilbig et al., 2019). At this regard, none of the 44 *Lat. sakei* cultures herein assayed showed the production of sucrose-independent EPS, whereas 21 isolates showed the formation of mucoid colonies in the presence of sucrose, thus suggesting a good *in vitro* synthesis of EPS to be further investigated.

Regarding the acid production capacity, and the capability to cope with the main environmental stresses encountered during the fermentation process (pH, NaCl, sodium nitrite, and potassium nitrate), the good performances of the tested *Lat. sakei* isolates inoculated in synthetic media were in accordance with those reported by Wen et al. (2021) for the same lactic acid bacteria species, thus suggesting their potential application in salami production for the formulation of starters or adjunct cultures. It is noteworthy that the acidification occurring in fermented sausages affects proteolysis and thus flavor formation. At this regard, fermented sausages produced in Northern European countries are characterized by a pH drop below 5.0 from fermentation throughout ripening, whereas in those produced in Southern European countries a moderate drop in pH is usually observed during fermentation, with final pH values comprised between 5.5 and 6.0 (Berardo et al., 2017). Hence, almost all the isolates herein assayed could serve as suitable acidifiers in fermented meat products.

Among the enzymatic activities observed in the selected pool of *Lat. sakei* isolates, the most relevant were leucine arylamidase and valine arylamidase activities observed for most of the isolates. The presence of these two aminopeptidases has already been reported in *Lat. sakei* strains. Of note, these enzymatic activities contribute to the hydrolysis of the sarcoplasmic proteins with the formation of free amino acids, these latter representing precursors of favor compounds in fermented sausages (Wang et al., 2013). The results on the presence of aminopeptidases obtained in the present study are in accordance with those reported by Ammor et al. (2005) that observed a strong leucine and valine arylamidase but no cystine arylamidase activity in *Lat. sakei* isolated from traditional French dry sausages.

In the present study, 27 *Lat. sakei* isolates showed a high acid phosphatase activity. Of note, this hydrolase liberates phosphate ions from organic esters at pH values ranging from 4.5 to 6.0. These results are in accordance with those reported by Papamanoli et al. (2003) that observed a high acid phosphatase activity in *Lat. sakei* cultures isolated from a Greek dry-fermented sausage.

As a positive trait, the absence of beta-glucuronidase in all the tested *Lat. sakei* isolates deserves attention. Indeed, the activity of this enzyme leads to the production of carcinogens and mutagens in the colon of the consumer, thus also increasing the probability of tumor induction (Li et al., 2023b).

Of note, the qPCR analyses showed negative results for the presence of the *hdcA* gene, thus attesting the absence of histidine decarboxylases in all the *Lat. sakei* isolates. However, although tyrosine decarboxylases are not widely distributed amongst bacteria (Dong et al., 2021), the isolates should further be screened also for the presence of tyrosine decarboxylase genes. Interestingly, as reported by Dong et al. (2021),

tyrosine decarboxylase negative strains of *Lat. sakei* isolated from Harbin dry sausage were able to degrade tyramine in synthetic growth medium, thus suggesting the use of this species in biological reduction of this biogenic amine.

In the analyzed *Ciauscolo* PGI salami, *D. algida* and *Leuc. carnosum* were also detected among the major pro-technolgical taxa.

D. algida (basonym *Lactobacillus algidus*) has already been detected by Belleggia et al. (2020a) in *Ciauscolo* PGI salami. The occurrence of *L. algidus* has also been reported by Greppi et al. (2015) in traditional Piedmontese sausages. This lactic acid bacterium represents a fastidious microorganism in meat since it can be the causative agent of spoilage with production of biogenic amines (Säde et al., 2020). Raw meat contaminated by *L. algidus* has a higher sour smell (Schirmer et al., 2009); notwithstanding, when a sour smell is produced in fermented sausages, this could not necessarily represent a negative trait.

As for *Leuc. carnosum*, the occurrence of this species has already been reported by Belleggia et al. (2020a) in *Ciauscolo* PGI salami. As reported by many authors, *Leuc. carnosum* can produce anti-listerial bacteriocin (leucocin) with a broad spectrum of activity (Hornbæk et al., 2004; Osmanağaoğlu, 2007; Woraprayote et al., 2021), thus suggesting the use of selected *Leuc. carnosum* isolates as protective adjunct cultures in *Ciauscolo* PGI manufacturing.

A few eumycetes species characterized the mycobiota of the *Ciauscolo* PGI salami herein analyzed.

The presence of *C. cladosporioides* in *Ciauscolo* PGI salami has already been reported by Belleggia et al. (2020a), and by Vila et al. (2019) among the minor fraction of filamentous fungi isolated from dry-cured sausages produced in Argentina. This species, usually occurring on the surface of fermented sausages, can originate from contaminated air and raw materials (Parussolo et al., 2019). As reported by Lozano-Ojalvo et al. (2015), *Cladosporium* species are generally not able to heavily colonize dry-cured meat products, although some xerotolerant and halotolerant strains can produce black spots on the surface of the sausages.

D. hansenii is one of the most widely detected yeast species in fermented sausages, and it contributes to the development of the flavour of fermented meat products through the production of endo- and exo-peptidases (Flores and Toldrá, 2011; Medina-Córdova et al., 2018). Indeed, the enzymatic activities carried out by *D. hansenii* enrich the meat matrix in free amino acids and peptides that characterize the aroma of the end product (Flores and Toldrá, 2011). Furthermore, the enzyme glutaminase produced by *D. hansenii* neutralizes the acidic pH of fermented sausages and generates L-glutamate that can act as flavor enhancer (Flores and Toldrá, 2011). Interestingly, in the last decade, *D. hansenii* attracted the attention of the fermented meat industry as bioprotective agents against toxigenic penicillia (Núñez et al., 2015).

Regarding *K. zeylanoides* (formerly known to as *Candida zeylanoides*), the presence of this yeast species has already been reported by Belleggia et al. (2020a) in *Ciauscolo* PGI salami and in fermented sausages produced in Italy (Giarratana et al., 2014) and other Southern European countries (Belleggia et al. 2020b, 2022a, 2022b; Encinas et al., 2000).

The SPME-GC/MS analysis performed during ripening of *Ciauscolo* PGI salami allowed the composition of the major and minor volatile components to be disclosed. In more detail, the presence of spices-derived components (mono- and sequiterpene hydrocarbons, sulphides and disulphides), detected throughout the whole ripening time of samples was in accordance with the results reported by other authors for different kinds of Italian salami (Bianchi et al., 2007; Jerković et al., 2010; Moretti et al., 2004). Particularly, the presence of the major aliphatic sulphur compounds (allyl methyl sulphide and diallyl disulphide) detected in the analyzed samples can likely been explained by allicin decomposition, that was recognized as main character in fresh garlic smell.

Hexanal was the most abundant lipid oxidation marker, thus confirming the results already observed in other Italian salami (Bianchi et al., 2007; Moretti et al., 2004). Interestingly, a marked decrease of hexanal in batch 1 samples came with an increase of hexanol, probably due to the reducing conditions inside the product.

Branched chain alcohols (3-methyl-1-butanol, 2-methyl-1-butanol) were the most represented markers of microbial activity, together with ethyl esters of isopentanoic, hexanoic (the most abundant), and octanoic acids (Chaves-López et al., 2011; Janssens et al., 2012) contributing to fruity notes of salami aromas. In fact, the esterifying activities of several yeasts, molds, and bacteria usually present in fermented meats have previously been reported (Bianchi et at., 2007; Lorenzo et al., 2013).

Only a limited number of aroma components herein detected could be related to microbial activity; among these, branched chain alcohols originated from amino acid catabolism (3-methyl-1-butanol, 2-methyl-1-butanol) were the most represented (Chaves-López et al., 2011; Janssens et al., 2012). Ethyl esters of isopentanoic, hexanoic (the most abundant), and octanoic acids were recognized as typical markers of microbial activity, as well. According to Bianchi et at. (2007) and Lorenzo et al. (2013), several yeasts, molds, and bacteria that are usually present in fermented meat could contribute to fruity notes of salami aromas through their esterifying activities.

Interestingly, a marked decrease of the main lipid oxidation product (hexanal) came with an increase of hexanol, probably due to the reducing conditions inside the product.

The correlation analysis allowed the contribution of the bacterial and fungal taxa in the volatilome profile to be hypothesized.

Of note, the positive correlation observed in the present study between *Lat. sakei* and ethyl isopentanoate (ester) and 3-methyl-1-butanol (with fruity and alcoholic flavor) has already been reported by Freiding et al. (2011), probably as a results of leucine metabolism. Freiding et al. (2011) also observed the production of 3-methyl-1-butanol by *Lat. curvatus* that, in the present study, was detected as minority species.

The positive correlation observed between *D. algida* and allyl-methyl disulfide suggests the contribution of this species in allicin decomposition.

The positive relationship observed in the present study between *Lac. plantarum* and ethyl pentanoate has already been reported by Hu et al. (2020) in traditional dry sausages from Northeast China.

Interestingly, beta-caryophillene, that was postitively correlated with *D. hansenii*, possesses considerable anticancer activities, affecting growth and proliferation of cancer cells (Fidyt et al., 2016). Hence, the occurrence of such positive correlation suggests further investigation to elucidate the role of *D. hansenii* in beta-caryophillene production in fermented sausages.

Finally, the positive correlation between *K. zeylanoides* and 2-methyl-1-butanol suggests a role of this yeast in the degradation of branched-chain amino acids that are the precursors of such volatile compound (Zhang et al., 2019).

5. Conclusions

The results of the present study provided an advancement of knowledge of the morpho-textural, microbiological, and volatile features of *Ciauscolo* PGI salami. As for colour and texture characteristics, for the first time this research provided objective parameters to be used as product quality indices. Regarding microbiota characterization, the dominance of *Lat. sakei* in the analyzed sausages was once again confirmed, together with other co-occurring species, namely *D. algida* and *Leuc. carnosum*. In the analyzed samples, the absence of pathogenic microorganisms attested the high quality of the raw materials and the production process, thus confirming *Ciauscolo* PGI salami as an Italian food excellence.

The pool of *Lat. sakei* cultures isolated from the analyzed samples showed a suitable acid production capacity, together with the capability of coping with the main environmental stresses occurring in fermented sausages. For most of the isolates, a strong aminopeptidase activity (due to leucine arylamidase and valine arylamidase) was observed.

Moreover, most of the isolates showed the *in vitro* production of sucrose-dependent EPS. Interestingly, no isolate was positive for beta-glucuronidase activity. Finally, no isolate was positive for the *hdcA* gene. Based on these findings, a few candidates as starter or adjunct cultures, with potential use for product safety and quality improvement, were found, being the isolates *Lat. sakei* C5, C7, C11, C31, C45, C48, C53, C55, and C60. Further research is needed to assess their supposed pro-technological traits in trials for salami manufacturing as well as the occurrence of transferable antibiotic resistances.

To the authors' knowledge only one available study has previously investigated the volatilome of *Ciauscolo* PGI salami; hence, the similarity of the volatile pattern detected in the present study with that already published allows a draft of the volatile fingerprint of the *Ciauscolo* PGI salami to be drawn. The journey never stops.

CRediT authorship contribution statement

Andrea Osimani: Conceptualization, Writing - Review & Editing, Supervision, Resources. **Luca Belleggia:** Investigation, Formal analysis. **Cristian Botta:** Investigation, Formal analysis. **Ilario Ferrocino:** Investigation, Formal analysis, Writing - Original Draft, Resources. **Vesna Milanović:** Formal analysis. **Federica Cardinali:** Investigation, Formal analysis, Writing - Original Draft. **M. Naceur Haouet:** Investigation. **Cristiana Garofalo:** Formal analysis. **Massimo Mozzon:** Investigation, Formal analysis, Resources. **Roberta Foligni:** Investigation, Formal analysis. **Lucia Aquilanti:** Review & Editing, Resources.

Declaration of competing interest

The authors declare that NO conflict of interest exists.

Data availability

Data will be made available on request.

Acknowledgments

This project was supported by the *Agenzia* per *i Servizi nel Settore Agroalimentare delle Marche* (ASSAM) within the Project: Bio.Mi.Ma "*Biodiversità Microbica delle Marche nei processi di trasformazione delle produzioni regionali tradizionali*" (Grant: MIPAAF - Legge 194/2015).

The isolation and characterization of the lactic acid bacteria cultures were funded by the European Union – Next Generation EU. Project Code: ECS00000041; Project CUP: C43C22000380007; Project Title: Innovation, digitalization and sustainability for the diffused economy in Central Italy – VITALITY.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102582.

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