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

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

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Abstract

In the present study, microbial dynamics of naturally fermented *Ciauscolo* PGI salami were studied through culture-dependent and metataxonomic analyses. Moreover, physico-chemical and morpho-textural evaluation, together with the analysis of volatile organic compounds were also carried out. Cultures of *Latilactobacillus sakei* were isolated from the analyzed samples and characterized for: i) acidification activity; ii) enzymatic activity; iii) the production of EPS; iv) production of bacteriocins against *Listeria innocua*, utilized as surrogate for *Listeria monocytogenes*. *Pseudomonas* spp. represented the dominant taxon in the raw materials, whereas *L. sakei* was the dominant species from t_5, together with *Dellaglioa algida* and *Leuconostoc carnosum*. Among eumycetes, *Cladosporium cladosporioides*, *Debaryomyces hansen*ii, *Kurtzmaniella zeylanoides*, and *Malassezia restricta/globosa* were the most abundant yeasts occurring in all samples. The 44 *L. sakei* cultures isolated from the analyzed samples 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. Based on these evidence, a few candidate starter or adjuncts cultures, with potential use for product safety and quality improvement, were highlighted, namely *L. sakei* C5, C7, C11, C31, C45, C48, C53, C55, and C60. In the analyzed 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.

Keywords: fermented sausages, metataxonomic analysis, *Latilactobacillus sakei*, acidification, enzymatic activity.

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 produce the transformation of the raw meat into the end product. In more detail, as soon 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, Mikuš, & Kiš, 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 (Bellegia 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), with negative impact on the safety of fermented sausages (Fong, El-Nezami, & Po Sze, 2021).

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. 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 2,500 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 dynamics of the microbiota naturally occurring in *Ciauscolo* PGI salami manufactured by an artisanal producer located in the Marche Region and to isolate a pool of lactic acid bacteria with potential pro-technological features (starter or adjunct cultures). 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; and iii) the production of EPS.

As reported by 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 *L. 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 2.7% (w w^{-1}), ground black pepper ~0.4% (w w^{-1}), ground garlic ~0.1% (w w^{-1}), white wine (20 mL/kg), and 0.05% (w w^{-1}) potassium nitrate (E252) 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 50% (v/v). The drying was performed for 5 days under the following conditions: progressive temperature decrease, from 20 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 6 cm width, were produced (Fig. 1). Collection of samples was performed at 0, 5, 10, 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 during the same day of collection.

2.2. Physico-chemical analyses

144

145 A pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy) was used to determine pH values
146 of *Ciauscolo* PGI salami samples through direct insertion of the probe into the food matrix. The data, in duplicate for
147 each sample, were reported as mean ± standard deviation.
148 Total titratable acidity (TTA) was determined on 10 g of sample homogenized in 90 mL of distilled water, adjusted to a
149 pH value of 8.3 with a 0.1 N solution of NaOH used as a titrant. Percentage (%) TTA of lactic acid equivalents was
150 calculated as previously reported by Rampanti et al. (2023). TTA% was reported as mean ± standard deviation of two
151 replicates.
152 The amounts of acetic acid and lactic acid were assessed using Acetic Acid (Acetate Kinase Manual Format) test kit and
153 D-/L-Lactic Acid (D-/L-Lactate) (Rapid) test kit (Megazyme, Bray, Ireland). The data, in duplicate for each sample, were
154 expressed as g $100g^{-1}$ of the sample and reported as mean ± standard deviation.
155 The water activity (a_w) was assessed in accordance with the ISO 21807:2004 standard method using an Aqualab 4TE
156 apparatus (Meter Group, Pullman, USA). The data, in duplicate for each sample, were reported as mean ± standard
157 deviation.
158 Color measurements was performed using a Chroma Meter CR-200 (Minolta, Osaka, Japan) with a D65 illuminant. Color
159 was determined on 2 cm thick slices according to CIE L*a*b* system (L*, lightness; a*, redness/greenness; b*,
160 blueness/yellowness) (Belleggia et al. 2022b). Visual changes during ripening of the sausages were evaluated by
161 longitudinally cutting them (7 mm thickness) and imaging the cross sections with a scanner (ENVY 6200 Series, HP,
162 Palo Alto, CA, United States) (Dreher et al., 2021)
163 Cylindrical specimens of meat batter and sausages (height: 15 mm, diameter: 20 mm) were excised and then uniaxially
164 compressed twice with a CT3-4500 texture analyzer (Brookfield Engineering Laboratories Inc., Middleboro MA, USA)
165 equipped with a 36 mm diameter cylindrical probe (mod. TA-AACC36) at 1.5 mm/s using a non-destructive deformation
166 (40%) (Dreher et al., 2021). Specimens were positioned between the load cell and the fixture base table of the instrument.
167 A 4500 g load cell was used.
168

169 *2.3. Viable counts*

170

171 Viable counts were performed by mixing 10 g of each sample with 90 mL of sterile peptone water (1 g L^{-1} of
172 bacteriological peptone) homogenized using a Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for
173 3 min at 260 rpm. After homogenization, ten-fold serial dilutions were prepared, and viable counts of the following
174 microbial groups were evaluated: i) lactic acid bacteria on De Man Rogosa and Sharpe (MRS) agar added with 250 mg

175 L^{-1} of cycloheximide (250 mg L^{-1}) to inhibit the growth of eumycetes, with incubation at 37 °C for 48-72 h; ii) coagulase-
176 negative cocci on Mannitol Salt Agar (MSA), with incubation at 37 °C for 48-72 h; iii) Enterobacteriaceae on Violet Red
177 Bile Glucose Agar (VRBGA), with incubation at 37 °C for 24 h; iv) eumycetes on Rose Bengal Chloramphenicol Agar,
178 with incubation at 25 °C for 72-96 h. The results of two biological and three technical replicates were expressed as the
179 log of colony-forming units (cfu) per gram of sample and reported as mean ± standard deviation.
180 Finally, a miniVIDAS apparatus (Biomerieux, Marcy l'Etoile, France) was used to assess the presence/absence of *Listeria*
181 *monocytogenes* and *Salmonella* spp. through the enzyme-linked fluorescent assay (ELFA) method, in accordance with
182 the AFNOR BIO 12/11-03/04 and AFNOR BIO 12/16-09/05 standard methods, respectively (Haouet et al., 2017).
183
184 *2.4. Real-time PCR analysis for the detection of botulinic toxins genes*
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186 The presence/absence of *Clostridium botulinum* was assessed via multiplex real-time PCR as already described by
187 Belleggia et al. (2020a). *C. botulinum* was analyzed in accordance with the multiplex real-time PCR method of the Italian
188 National Reference Centre for botulism (http://old.iss.it/binary/cnrb/cont/CNRB31.010.pdf) for the detection of the target
189 genes *bont/A, bont/B, bont/E, bont/F, and 4gyrB (CP*) that codify for botulinic toxins.
190 Briefly, 25 g of *Ciauscolo* PGI salami sample were blended in 225 mL Triptone Peptone Glucose Yeast extract (Microbiol
191 Diagnostici, Italia), incubated in anaerobiosis at 30°C for 96 h and then extracted with 6% Chelex-100 (Biorad, Milan,
192 Italy). The amplification was carried out using the Kit QuantiTect multiplex No Rox (Qiagen) in the Stratagene Mx3005P
193 (Agilent Technologies) thermal cycler and the primers and probes nucleotide sequences are those already reported by
194 Belleggia et al. (2020a). Two different Master mixes were used concurrently, with the following thermal profile: 1 cycle
195 at 95°C for15 min followed by 40 cycles at 94°C for 30 s and 56°C for 90 s.
196
197 *2.5. RNA extraction and cDNA synthesis*
198
199 For each sample homogenate (dilution 10^{-1}), prepared as reported above, 1.5 mL aliquot was centrifugated at 16,000 rpm
200 for 10 min. RNA*later* Stabilization Solution (Ambion, Foster City, CA, USA) was used to protect the resulting cell pellets
201 that were subsequently stored at -80 °C. Quick-RNA MiniPrep kit (Zymo Research, Irvine, California, USA) was then
202 used to extract the total microbial RNA from the cell pellets in accordance with manufacturer's instructions. PCR
203 amplification with universal prokaryotic primers (27 f, 1495 r) (Weisburg et al., 1991) was carried out to check for the
204 presence of residual DNA. SensiFAST cDNA Synthesis Kit for RT-qPCR (Bioline, London, UK) was used to synthesize
205 cDNA according to manufacturer's instructions.

206

207 *2.6. Amplicon target sequencing and bioinformatic analysis*

208

209 The cDNA was used as template to amplify the V3-V4 region of the 16S rRNA gene for bacteria (Klindworth et al.,
210 2013), whereas the D1 domain of the 26S rRNA gene of Large Ribosomal Subunit (LSU) was targeted for fungi (Mota-
211 Gutierrez et al., 2019).
212 A total of 400,802 and 535,306 raw-reads were produced by 16S and 26S amplicon-based sequencing, respectively. To
213 obtain Amplicon Sequence Variants (ASVs) the raw-reads were analyzed with *DADA2* package (Callahan et al., 2016)
214 in R environment (R version 4.1.1; http://www.r-project.org). The pipeline described by Botta et al. (2022) was applied
215 to filter and merge raw-reads: 157,311 paired-end bacterial reads (19,664 reads/sample) and 270,743 paired-end fungal
216 reads (45,124 reads/sample) passed the filtering parameters and were used to construct ASVs tables.
217 Taxonomy was assigned with a 99 % sequence similarity through Bayesian classifier method (Wang et al., 2007) by
218 matching bacterial ASVs to the 2021 release (version 138.1) of Silva prokaryotic SSU reference database
219 (https://zenodo.org/record/4587955#.YObFvhMzZRE). Fungal ASVs taxonomy was assigned at 99 % against an internal
220 database of 26S rRNA (Mota-Gutierrez et al., 2019). Taxonomy assignment for 16S and 26S was double checked by
221 using BLASTn suite (https://blast.ncbi.nlm.nih.gov).
222 Fungal and bacterial ASVs were aligned with *DECIPHER* package and two unrooted phylogenetic tree were constructed
223 with *phangorn* package (Schliep, 2011; Wright, 2016). Alpha diversity metrics and weighted UniFrac beta-diversity
224 distance were calculated with *phyloseq* and *picante* packages (Kembel et al., 2010; McMurdie & Holmes, 2013):
225 rarefaction limit was set to the lowest number of sequences/sample.

226

227 *2.7. GC-MS analysis of volatile components*

228

229 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
230 in a thermostatic bath. The static headspace was sampled by a 65 μm PDMS/DVB SPME fibre (Supelco, Bellefonte, PA)
231 for 15 minutes. Chromatographic separation of volatiles was performed by a fused silica capillary column ZB-5 (30 m L,
232 0.25 mm ID, 0.25 μm FT; Phenomenex, Torrance, CA) mounted on a Varian 3900 gas chromatograph (Varian Analytical
233 Instruments, Walnut Creek, CA); the injector was operating in splitless mode for 0.1 min at a constant temperature of 250
234 °C; oven temperature was increased from 40 °C to 220 °C at a rate of 6 °C/min, then held at the final temperature for 5
235 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, Walnut Creek, CA): 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) 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 L. sakei

2.8.1. Isolation and identification

As previously described, colonies of lactic acid bacteria grown on MRS agar added with cycloheximide were randomly selected and subsequently sub-cultured to purity under the same conditions. 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 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 Laboratories, Hercules, CA, USA) 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 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, Ohio, 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 analysed with UCHIME2 software tool to uncover chimeras (Edgar, 2016) and were 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

L. sakei isolates were sub-cultured twice in MRS broth incubated at 30 °C for 18 h (Wen et al., 2021). The cultures were centrifuged at 4,000 rpm for 5 min 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 spectrophotometer (Shimadzu UV-1800, 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; ii) MRS broth added with 150 mg Kg^{-1} of E250 (sodium nitrite); iii) MRS broth added with 150 mg Kg^{-1} of E252 (potassium nitrate); iv) MRS broth added with 3% (w v^{-1}) of NaCl; v) MRS broth added with 150 mg Kg^{-1} of E250, 150 mg Kg^{-1} of E252, and 3% (w v^{-1}) of NaCl. The pH values of the media were measured prior to inoculation (t_0) and after incubation at 30 °C for 0, 4, and 24 h.

2.8.3. Semi-quantitative assessment of enzymatic activities

The semi-quantitative micromethod API® ZYM (bioMérieux, Marcy-l'Etoile, France) 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 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 (2 mL) of each isolate grown on MRS agar was prepared according to a turbidity of 5-6 McFarland. Sixty-five µL of the obtained suspension were used for the inoculation of each cupule of the API® ZYM strips with incubation for 4 hours at 37°C. After incubation, 1 drop of ZYM A reagent and 1 drop of ZYM B reagent were added to each cupule until colour development (at least 5 minutes). 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

297 The antimicrobial activity of the selected pool of isolates ascribed to *L. sakei* was performed by following the agar well
298 diffusion assay, described by Parente et al. (1995). Briefly, molten Brain Heart Infusion (BHI) soft agar (0.75% agar)
299 (VWR Prolabo Chemicals) was inoculated (2%, v v^{-1}) with the indicator microorganism *Listeria innocua*. Twenty mL of
300 the inoculated medium were poured into 90 mm Petri dish and left to solidify. A cone of a 200 µL sterile tip was utilized
301 to create wells of ~ 50 µL capacity on BHI soft agar. Previously, lactic acid bacteria were sub-cultured twice in MRS
302 broth at 37 °C for 48 h. The broth cultures were then added with 0.1 N NaOH solution to reach pH 7.0 to neutralise the
303 organic acids produced during the bacterial growth. A filtration step on sterile PES membrane filter of 0.22 µm pore size
304 (Laboindustria S.p.A., Padova, Italy) followed. For each lactic acid bacteria isolate, 4 wells were formed on BHI soft
305 agar, each containing: (i) 50 µL of the sub-cultured suspension; (ii) 50 µL of the neutralised suspension adjusted to pH
306 7.0; (iii) 50 µL of the filtered neutralised suspension; (iv) 50 µL of sterilised water as a negative control. Afterwards, the
307 Petri dishes were incubated at 37 °C for 24 h and examined for the presence of zones of inhibition. In the case of positive
308 results (presence of inhibition halo), 3 spots of 5 µL each of pepsin (Fluka™, Honeywell, Morristown, USA), trypsin
309 (Fluka™) or Pronase (Merck KGaA, Darmstadt, Germany) were set along the circumference of the inhibition zone to
310 evaluate the protein nature of the antimicrobial. The Petri dishes were further incubated at the same conditions. The
311 antimicrobial activity of lactic acid bacteria isolates due to the synthesis of bacteriocins was confirmed by the formation
312 of crescents.
313
314 *2.8.5. In-vitro EPS production*
315 The selected pool of isolates ascribed to *L. sakei* was screened for EPS production, based on the method already reported
316 by Hilbig et al. (2019) with some modifications. In more detail, the isolates were retrieved from cryo-protective
317 suspensions and sub-cultured twice on MRS broth at 37 °C for 48 h. The EPS production was visually observed by adding
318 aliquots (5 µL) of each bacterial culture on the following solid media: (i) MRS agar supplemented with sucrose (80 g L^{-1}) to promote the synthesis of homopolysaccharides (HoPS); MRS agar supplemented with yeast extract (10 g L^{-1}), meat
320 extract (10 g L^{-1}), galactose (20 g L^{-1}), and lactose (20 g L^{-1}) to promote the synthesis of heteropolysaccharides (HePS).
321 After an incubation period of 48 h at 30 °C, the colonies were classified as positive whenever presenting a mucoid aspect
322 (visible shiny and slimy appearance) or a ropy consistence (able to produce detectable filaments by using a sterile
323 toothpick). For each isolate, the analyses were conducted in duplicate.
324
325 *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 volatile organic compounds (VOCs) was performed by means of Spearman's rank correlation.

3. Results

3.1. Physico-chemical analyses

The results of physico-chemical analysis 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 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 100g^{-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 Figure 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.

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

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. could be detected in 25 g of product, irrespective of the sampling time.

3.4. Metataxonomic analyses

3.4.1. Microbiota

Microbiota of *Ciauscolo* PGI salami differed significantly (PERMANOVA, *P*[FDR]<0.05) if the day of production (t_0) was compared to the rest of ripening period in both batches (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 along time (data not shown).

By considering the composition of the microbiota, at t_0, in both batches, three *Pseudomonas* species s (*P. fragi, P. psychrophila, P. lundensis*) represented the dominant taxa (Fig. 3). From t_5, *L. 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 five species of yeast and one genus of mold that cumulatively represented more than 50% of the relative abundance in all samples was observed (Fig. 4, Supplementary Table 2). In fact, *Cladosporium cladosporioides*, *Debaryomyces hansen*ii, *Kurtzmaniella zeylanoides* and *Malassezia restricta/globosa* were the most abundant yeasts occurring in all samples, followed by less ubiquitous ASVs. Filamentous fungi were mainly represented by *Penicillium* sp., which was 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 L. sakei isolates

The BLAST search allowed the unambiguous identification of 44 *L. 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 *L. 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, this 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 reported in Table 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 and 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 (4.9), whereas the isolates C8, C9, C17, C19, C31, C34, C37, and C4 0reached the lowest pH values (4.1) 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 *L. sakei* C55 showed the highest pH after 24 (4.9), whereas the medium inoculated with the isolates C8, C15, C17, C21, and C29 reached the lowest pH (4.1) 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 (3.9) 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 (4.7). By contrast, the growth medium inoculated with the isolates C17, C29, C31, or C37 reached the lowest pH (3.9) after 24 h.

As for MRS broth added with 150 mg Kg^{-1} of E250, 150 mg Kg^{-1} of E252, and 3% NaCl, according to ANOVA results (data not shown), the isolate C2 showed the lowest pH drop after 24 h (5.0), whereas the medium inoculated with the isolate C8 reached the lowest pH (3.9) 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 Figure 5.

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 *L. 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. 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.

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 6.

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, we observed patterns of positive and negative correlations (*P*-value [FDR adjusted] <0.05) mainly influenced by the batch and then by the sampling time (**Fig_X**). *L. sakei* was negatively correlated with cis-sabinene hydrate and positively with to ethyl isopentanoate (ester) and with 3-methyl-1-butanol together with *L. curvatus* while *Dellaglioa algida*, with allyl-methyl disulfide and α-thujene. L. plantarum showed positive relationship with ethyl pentanoate and 1-pentanol.

Regarding fungi we observed a direct relationship between *Debaryomyces hansenii* and *Kurtzmaniella zeylanoides* with β-caryophillene and 2-methyl-1-butanol (alcohol), respectively.

[modify in relation to the discussion]

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-

477 textural, physico-chemical (including volatile compounds), and microbiological characteristics of *Ciauscolo* PGI salami
478 during ripening were investigated. *L. sakei* isolates collected from the analyzed samples were also tested for some pro-
479 technological activities to select potential cultures to be used as starter or adjuncts.
480 As for pH values detected in the analyzed samples, the data were in accordance with those already detected by Belleggia
481 et al. (2020a) and Trani et al. (2010) in *Ciauscolo* salami produced by other manufacturers. In the analyzed samples, a
482 progressive decrease in pH was observed during time, irrespective of the production batch. Of note, since *Ciauscolo* is
483 ready for consumption only after 20 days, the pH values herein detected in the end products reflect the short ripening
484 time. According to the production disciplinary, the pH of ready-to-eat *Ciauscolo* PGI salami should be $\geq$ 4.8. In
485 accordance with pH reduction, an increase in TTA values as well as in lactic and acetic acid content was also observed in
486 the analyzed samples. The trends of the abovementioned parameters can likely be explained by the activity of the lactic
487 acid bacteria naturally occurring in the raw material which produced organic acids through fermentative metabolism of
488 meat carbohydrates (Fadda, López, & Vignolo). Of note, the presence of both lactic and acetic acid in the analyzed
489 samples attests the presence of homofermentative as well as heterofermentative lactic acid bacteria.
490 In the analyzed samples, a reduction of a_w was observed during time, thus attesting the progressive drying of the product
491 with further improvement of the safety and stability of *Ciauscolo* PGI salami. The detected values were in accordance
492 with those detected by Trani et al. (2010) and Belleggia et al. (2020a) in the same type of fermented sausage.
493 Interestingly, pH and a_w values herein detected were higher than those detected by Aquilanti et al. (2007) in the same type
494 of fermented sausage, , thus suggesting a great variation of these parameters based on the artisan method applied during
495 production.
496 Based on Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, ready-to-eat products
497 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 *Listeria*
498 *monocytogenes*, hence, such issue should be taken into consideration when producing *Ciauscolo* salami, whose pH and
499 a_w values can sometimes be permissive for the growth of the pathogen.
500 For color measurements, it is noteworthy that *Ciauscolo* PGI salami is a short-time ripened fermented sausage, hence, the
501 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
502 knowledge, no data reporting the color of *Ciauscolo* PGI salami are available in the scientific literature for further
503 comparison of results. Regarding lightness, this parameter can vary from 0 (black) to 100 (white) (Méndez-Cid et al.,
504 2017); hence, the high level of lightness detected in the samples herein analyzed can likely be attributed to the high
505 amount of fat that characterizes the meat batter of this fermented sausage (Estévezet al., 2005). As for a* parameter, this
506 axis represents the green–red opponent colors, with values < 0 toward green and values > 0 toward red (Méndez-Cid et
507 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 higher the measured hardness. This evidence was also supported by the lower a_w values measured in the samples a 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) 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 & 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. Enterobactericeae, together with coliforms, are often assessed in foods to highlight poor hygiene or inadequate processing, process failure, and 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

539 acid) produced by lactic acid bacteria prevent the development of spoilage and pathogenic microorganisms. Of note, some
540 strains of lactic acid bacteria can act as protective cultures in fermented meat products through the production of
541 bacteriocins. These compounds are proteins with potential antimicrobial activity against specific pathogens (e.g., *Listeria*
542 *monocytogenes*) (Junges da Costa et al., 2021). Moreover, lactic acid bacteria metabolism leads to the stabilization of
543 meat color and to texture development (e.g., formation of a distinctive gel-like texture due to protein denaturation ()
544 (Fadda et al., 2010). Lactic acid bacteria are also responsible for production of volatile compounds through the release of
545 free amino acids (Fadda et al., 2010). In the present study, the counts of lactic acid bacteria were consistent with those
546 already detected by Belleggia et al. (2020a) and Aquilanti et al. (2007) in the same salami at 20 days of ripening, attesting
547 at about 8 log cfu g^{-1}.
548 In fermented meat, coagulase-negative cocci enhance the aroma and texture of the product. In more detail, this microbial
549 group encompasses microorganisms that produce nitrate reductase, this latter enzyme converting nitrate into nitrite
550 (Khusro, & Aarti, 2022). Nitrites exert an antimicrobial activity against clostridia, especially *C. botulinum*, with a
551 consequent reduction of food poisoning risk; they also exert an antioxidative activity and promote the formation of
552 nitrosomyoglobin with the subsequent development and stabilization of a pleasant red color (Khusro, & Aarti, 2022).
553 Furthermore, coagulase-negative cocci improve flavor and aroma of fermented sausages through the formation of volatile
554 compounds deriving from proteolysis and lipolysis (Khusro, & Aarti, 2022). In the present study, an increase in coagulase-
555 negative cocci counts was observed during time, with final counts that were similar to those already detected by Aquilanti
556 et al. (2007) in *Ciauscolo* PGI at 20 days of ripening, but higher than those recently detected by Belleggia et al. (2020a)
557 in the same salami, thus suggesting the occurrence of a certain variability among producers based on production process
558 and the raw materials used.
559 As for eumycetes (yeasts and molds), their role in fermented sausages is mainly related to the production of proteolytic
560 (exoproteases) and lipolytic (exolipases) enzymes that are responsible for the development of flavor and aroma (Copetti,
561 2019; Sunesen & Stahnke, 2003). Of note, yeasts are common environmental contaminants of fermented sausages and
562 are well adapted to this high-salt and acidic environment (Osei Abunyewa, Laing, Hugo, & Viljoen, 2000). As reported
563 by Cocolin et al. (2011), molds occurring on the surface of fermented sausages facilitate the dehydration process, since
564 they create micro-pores on the casing. Finally, external mold layer protects lipids from oxidation in the presence of light
565 (Cocolin et al., 2011).
566 Of note, the absence of the major pathogens as *L. monocytogenes*, *Salmonella* spp., and of the target genes *bont/A, bont/B,*
567 *bont/E, bont/F,* and *4gyrB (CP*), encoding botulinic toxins, in all the samples herein analyzed attests the high quality of
568 the raw materials and the proper application of good manufacturing practices.

569 Metataxonomic analysis highlighted differences in the microbiota of the analyzed samples, including stuffed meat batter,
570 fermented sausages sampled during ripening, and fermented sausages sampled at the end of maturation; in more detail, a
571 progressive substitution of meat spoilage bacteria of with pro-technological microorganisms was seen.
572 *Pseudomonas*, that encompasses psychotropic bacteria, constituted the core microbiota of stuffed salami at t_0. *P. fragi* is
573 a spoilage microorganism that produces slime on meat during storage and it is responsible for off-odors and soften of the
574 flesh (Shao et al. 2021). *P. psychrophila* is a facultatively psychrophilic bacterium that can be responsible for meat
575 spoilage (Yumoto et al., 2001). Regarding *P. lundensis*, this is a further spoilage species that has already been isolated
576 from beef and lamb fresh meat, where it was responsible for the production of off-odors (Shao et al. 2021). As reported
577 by Fidan et al. (2022), the presence of organic acids (as lactic or acetic acid) can inhibit putrefactive bacteria, including
578 those belonging to the genus *Pseudomonas*, thus explaining the reduction of such microorganisms as the fermentation of
579 the sausages herein analyzed has progressed.
580 As for the most represented pro-technological species, *L. sakei* and *L. carnosum* dominated in salami of batch 1 after 5
581 days of ripening, whereas the same two species, together with *D. algida*, were mostly represented in samples of batch 2.
582 *L. sakei* represents the emblematic lactic acid bacteria species in fermented meat products for its rapid acid production
583 (Amadoro, Rossi, Piccirilli & Colavita, 2015; Zagorec & Champomier-Vergès, 2017). Indeed, this psychrotrophic and
584 facultative anaerobic species has already been recognized as the key lactic acid bacterium in fermented sausages produced
585 in Western Europe, as well as in fermented meat products manufactured in Asia and South America (Zagorec &
586 Champomier-Vergès, 2017). In meat or fermented sausages, *L. sakei* takes advantage of meat nutrients by encoding
587 oligopeptide transporters and intracellular peptidases through genes up-regulation. The endo and exo-peptidases produced
588 by *L. sakei* increase the concentration of free amino acids, thus positively affecting flavor development (Belleggia et al.,
589 2022b). Moreover, *L. sakei* can use the ribose present in raw meat as carbon source through an ATP-dependent system
590 (Zagorec & Champomier-Vergès, 2017). Based on the abovementioned features, in fermented sausages, *L. sakei* shows a
591 shorter lag phase and a growth rate higher than other lactic acid bacteria; moreover, it is highly salt tolerant (up to 6.5%
592 NaCl) (Amadoro et al., 2015).
593 As reviewed by Honrada Perez, Zendo, & Sonomoto (2022), *L. sakei* can produce multiple bacteriocins, namely sakacin
594 P, sakacin T, and sakacin X, depending on the strain. At this regard, none of the 44 *L. sakei* cultures herein isolated
595 showed an antimicrobial activity against *Listeria innocua*. The production of bacteriocins represents a highly desirable
596 feature due to the competitive advantage obtained by the producing strain and for the increased safety of the product. Of
597 note, the genes encoding for active bacteriocins are frequently located in operon clusters, harbored in the genome, plasmid,
598 or in other mobile genetic elements; moreover, the expression of these operons is complex and can be induced by the
599 presence of auto-inducer peptides (Kumariya et al., 2019). The abovementioned features might explain the lack of

600 bacteriocins production in the analyzed *L. sakei* isolates and suggest that bacteriocin production by *L. sakei* is less
601 common than expected as already reported by Belleggia et al. (2022c), who observed no listericidal effect of 22 *L. sakei*
602 cultures isolated from fermented fish sausages.
603 As reported by Wang, Song, Zhao, Han, & Zhou (2019), strains of *L. sakei* also showed a high-yield EPS production with
604 unique characteristics for their exploitation in large-scale industrial food applications. EPS produced in fermented
605 sausages by lactic acid bacteria can impact on the morpho-textural properties of the end product (Hilbig et al., 2019). At
606 this regard, none of the 44 *L. sakei* cultures herein assayed showed the production of sucrose-independent EPS, whereas
607 21 isolates showed the formation of mucoid colonies in the presence of sucrose, thus suggesting a good *in vitro* synthesis
608 of EPS to be further investigated.
609 Regarding the acid production capacity, and the capability to cope with the main environmental stresses encountered
610 during the fermentation process (pH, NaCl, sodium nitrite, and potassium nitrate), the good performances of the tested *L.*
611 *sakei* isolates inoculated in synthetic media were in accordance with those reported by Wen et al. (2021) for the same
612 lactic acid bacteria species, thus suggesting its potential application in salami production for the formulation of starter or
613 adjunct cultures.
614 In is noteworthy that the acidification occurring in fermented sausages affects proteolysis and thus flavor formation. At
615 this regard, fermented sausages produced in Northern European countries are characterized by a pH drop below 5.0 from
616 fermentation throughout ripening, whereas in those produced in Southern European countries a moderate drop in pH is
617 usually observed during fermentation, with final pH values comprised between 5.5 and 6.0 (Berardo et al., 2017). Hence,
618 almost all the isolates herein assayed could serve as suitable acidifiers in fermented meat products.
619 Among the enzymatic activities observed in the selected pool of isolates ascribed to *L. sakei*, the most relevant were
620 leucine arylamidase and valine arylamidase activities showed by most of the isolates. The presence of these two
621 aminopeptidases has already been reported in *L. sakei* strains. Of note, these enzymatic activities contribute to the
622 hydrolysis of the sarcoplasmic proteins with the formation of free amino acids, these latter representing precursors of
623 favor compounds in fermented sausages (Wang et al., 2013). The results on the presence of aminopeptidases obtained in
624 the present study are in accordance with those reported by Ammor et al. (2005) that observed a strong leucine and valine
625 arylamidase but no cystine arylamidase activity, in *L. sakei* isolated from traditional French dry sausages.
626 In the present study, 27 isolates showed a high acid phosphatase activity. Of note, this hydrolase liberates phosphate ions
627 from organic esters at pH values ranging from 4.5 to 6.0. These results are in accordance with those reported by
628 Papamanoli et al. (2003) that observed a high acid phosphatase activity in *L. sakei* cultures isolated from a Greek dry-
629 fermented sausage.

As a positive trait, the absence of beta-glucuronidase in all the tested *L. 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., 2023).

In the analyzed *Ciauscolo* PGI salami, *D. algida* and *L. 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 be *L. algidus* has a higher sour smell (Schirmer, Heir, & Langsrud, 2009); notwithstanding, when a sour smell is produced in fermented sausages, this could not necessarily represent a negative trait.

As for *L. carnosum*, the occurrence of this species has already been reported by Belleggia et al. (2020a) in *Ciauscolo* PGI salami. As reported by many authors, *L. carnosum* can produce anti-listerial bacteriocin (leucocin) with a broad spectrum of activity (Hornbæk et al., et al., 2004; Osmanağaoğlu, 2007; Woraprayote et al., 2021), thus suggesting the use of selected *L. carnosum* isolates as protective adjunct culture in *Ciauscolo* 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 of eumycetes, 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 (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 & 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 & 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 & 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; Belleggia et al., 2022a; Belleggia et al., 2022b; Encinas, López-Dıáz, Garcıá-López, Otero, & Moreno, 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 kind of Italian salami (Bianchi et al., 2007; Jerković et al., 2010; Moretti at 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 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 cured 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 cured 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.

5. Conclusions

The results of the present study provided an advancement of knowledge on the morpho-textural, microbiological, and volatile features of *Ciauscolo* PGI salami. As for colour and texture characteristics, for the first time this research provides objective parameters to be used as product quality indices. Regarding microbiota characterization, the dominance of *L.*

692 *sakei* in the analyzed sausages was once again confirmed, together with other co-occurring species, namely *Dellaglioa*
693 *algida* and *Leuconostoc carnosum*. The pool of *L. sakei* cultures isolated from the analyzed samples showed a suitable
694 acid production capacity, together with the capability of coping with the main environmental stresses occurring in
695 fermented sausages. For most of the isolates, a strong aminopeptidase activity (due to leucine arylamidase and valine
696 arylamidase) was observed. Moreover, most isolates showed the *in vitro* production of sucrose-dependent EPS.
697 Interestingly, no isolate were positive for beta-glucuronidase activity. Based on these findings, a few candidates as starter
698 or adjuncts cultures, with potential use for product safety and quality improvement, were found, being the isolates *L. sakei*
699 C5, C7, C11, C31, C45, C48, C53, C55, and C60. Further research is needed to assess their supposed pro-technological
700 traits in trials for salami manufacturing. The absence of pathogenic microorganisms attests the high quality of the raw
701 materials and the production process, thus confirming *Ciauscolo* PGI salami as an Italian food excellence. To the authors'
702 knowledge only one available study has previously investigated the volatilome of *Ciauscolo* PGI salami; hence, the
703 similarity of the volatile pattern detected in the present study with that already published allows a kind of volatile
704 fingerprint of the *Ciauscolo* PGI salami to be drawn.
705
706 **CRediT authorship contribution statement**
707
708 **Andrea Osimani:** Conceptualization, Writing - Review & Editing, Supervision, Resources. **Luca Belleggia**:
709 Investigation, Formal analysis. **Ilario Ferrocino**: Investigation, Formal analysis, Writing - Original Draft, Resources.
710 **Cristian Botta**: Investigation, Formal analysis. **Vesna Milanović**: Formal analysis. **Federica Cardinali**: Investigation,
711 Formal analysis, Writing - Original Draft. **M. Naceur Haouet**: Investigation. **Cristiana Garofalo**: Formal analysis.
712 **Massimo Mozzon**: Investigation, Formal analysis, Resources. **Roberta Foligni**: Investigation, Formal analysis. **Lucia**
713 **Aquilanti**: Review & Editing, Resources.
714
715 **Declaration of Competing Interest**
716
717 The authors declare that they have no known competing financial interests or personal relationships that could have
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719
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721

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Data availability

Data will be made available on request.

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FIGURE CAPTIONS

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

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.

Fig. 3. Microbiota composition.

PCoA charts (on the left) displaying weighted UniFrac distance matrix (β-diversity). Batches and sampling time are defined by different colours 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.

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.

Fig. 5. Heat map representing the results of semi-quantitative assessment of enzymatic activities of *Latilactobacillus sakei* isolated (C1-C60) 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 (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).

Fig X. Correlation between metataxonomic and volatolomic 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 more to the less abundant, whereas the VOCs are grouped in relation to their chemical class (refer to the caption for the codes). Colours represents 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).