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Irene Franciosa [1,2], Monika Coton [2], Ilario Ferrocino [1], Maria Rita Corvaglia [1], Elisabeth Poirier [2], Jean-Luc Jany [2], Kalliopi Rantsiou[1], Luca Cocolin [1] and Jérôme Mounier [2]*

[1] University of Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Largo Braccini 2, 10095 Grugliasco, Torino, Italy

[2] Univ Brest, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, F-29280 Plouzané, France

*corresponding author: phone: +33 290915121, fax: +33 290915101, email: jerome.mounier@univ-brest.fr

Abstract

Aims: The complex mycobiota that colonizes traditional fermented sausages plays an important role in the organoleptic properties of such products. The aim of the present study was to investigate fungal diversity and mycotoxin production during maturation of PGI *Salame Piemonte*.

Methods and Results: Casing and meat samples were collected at five sampling times from three different batches produced in the same factory and analysed using culture-dependent and -independent approaches. *Penicillium nalgiovense,* which was deliberately inoculated, and *Debaryomyces hansenii,* were the most dominant taxa in casings. Several other fungi mainly belonging to *Penicillium crustosum*, *P. glabrum, P. nordicum*, *Cladosporium* spp*., Candida sake, C. zeylanoides* and *Yarrowia divulgata* were also identified. The casing mycobiota was compared to that of the meat using a metataxonomic approach and a higher fungal diversity was observed in meat as compared to casings. Mycotoxins and penicillin G were monitored using QTOF LC-MS and only trace amounts of roquefortine C were detected in two batches.

Conclusions: The present study highlighted the diversity of *Salame Piemonte* mycobiota and the important contribution of autochthonous fungi to its diversity. The absence of mycotoxins and penicillin G confirmed the high hygienic quality of the studied product regarding fungal and mycotoxin contamination.

Significance and Impact of Study: For the first time, this study provides insights about the *Salame Piemonte* mycobiota which together with the bacterial microbiota and *Salame Piemonte* process specifications are responsible for the product organoleptic properties.

Keywords: Fungi; Italian sausage; Culture-dependent approach; Metataxonomic approach; Extrolite detection; *Penicillium nalgiovense*

INTRODUCTION

Fermented meat products constitute a significant part of the Mediterranean diet and their production and commercialization contribute to the local economies (Baka *et al*. 2011). In the last decades, many studies have focused on the microbiota of fermented sausages (Ferrocino *et al*. 2018). However, most of the studies only focused on bacterial microbiota, *i.e*., lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS) that are the most important bacterial groups involved in the fermentation process (Franciosa *et al*. 2018). To date, fungal diversity and their functional roles in these products have been less studied (Toldrá *et al*. 2014).

Many fungal species are well adapted to the ecological conditions encountered during meat fermentation including intrinsic (pH, water activity, NaCl content, natural and intentionally added antimicrobials, available nutrients) and extrinsic factors (temperature, humidity) (Sonjak *et al*. 2011). That is why many fermented meat products, including fermented sausages, are characterized by fungal growth (both yeasts and molds) on the casing surface. Their metabolic activities, including glucidolytic, proteolytic and lipolytic activities, can be linked to aroma compound production that contribute to the typical cured flavours of the final products (Cruxen *et al*. 2019; Murgia *et al*. 2019; Parussolo *et al*. 2019; Vila *et al*. 2019). Other functional roles of desirable fungi include competition/exclusion of undesirable fungi (including mycotoxin producers) and surface colonization which changes the visual aspect of the products (Pitt and Hocking 2009). This latter role also helps to prevent excessive drying, protects against oxidative processes through peroxide metabolization and O_2 consumption, improves meat colour, increases pH via lactate utilization and deamination activities and facilitates sausage casing peeling through hyphae penetration into the casing (Comi and Iacumin 2013; Toldrá *et al*. 2014; Belleggia *et al*. 2020b).

Given the important functional roles played by this mycobiota, manufacturers often use selected fungal strains, especially the mold species *Penicillium nalgiovense* (Sunesen and Stahnke 2003) and the yeast species *Debaryomyces hansenii* (Murgia *et al*. 2019). These species are inoculated onto the casing surface to better control the fermentation and ripening process and improve overall product quality by ensuring more constant quality attributes (Meftah *et al*. 2018). The latter species are also frequently encountered in spontaneously fermented sausages (Montanha *et al*. 2018; Vila *et al*. 2019), but many other indigenous fungal species, including desirable and undesirables ones, can also be found in these products. Given the

positive impact fungal species can have on product quality, it is of interest to investigate fungal diversity to improve our knowledge on this kind of product.

As mentioned above, growth of undesirable mold species producing mycotoxins*, i.e.,* secondary metabolites toxic to human health, may also occur during the fermentation process (Pleadin *et al*. 2017; Meftah *et al*. 2018), especially in conditions where air quality control, among other factors, is unsatisfactory. The main mycotoxin producers encountered in fermented meats belong to *Aspergillus* and *Penicillium* spp. (Montanha *et al*. 2018). More specifically, the most common species are *Penicillium crustosum* that can produce penitrem A and roquefortine C (Coton and Dantigny 2019) and *Penicillium nordicum* and *Penicillium verrucosum* capable of producing ochratoxin A (OTA) (Iacumin *et al*. 2009; Samson *et al*. 2010). For the latter mycotoxin, the Italian Ministry of Health recommended, in meat or meat products, a maximum value of 1 µg/Kg of OTA (Ministero della Sanità 1999). It is worth mentioning that there is currently no European regulation specifying maximum mycotoxin concentrations in meat products in contrast to other foods, e.g., cereals and cereal-based products, groundnuts, oilseeds, dairy products, for which maximum levels are set by the European Union in EU Regulation 1881/2006 (Official Journal of the European Union 2006; Montanha *et al*. 2018). In addition to mycotoxins, several *Penicillium* species belonging to the *Chrysogena* section, including *P. nalgiovense*, are able to produce the antibiotic penicillin (Papagianni *et al*. 2007; Parussolo *et al*. 2019). Penicillin presence in food is undesirable as it can cause human allergies (Stone *et al.* 2020) and could also lead to resistance development in food-borne bacteria and/or commensal gut bacteria as well as the transfer of resistance genes to pathogenic bacteria (Verraes *et al*. 2013). Identifying fungal species that compose the fermented sausage casing mycobiota and quantifying potential hazardous secondary metabolites is therefore essential as part of safety risk assessment.

In this context, the aim of the present study was to investigate mycobiota diversity and dynamics of a typical fermented sausage, *Salame Piemonte*, granted with a Protected Geographical Indication (PGI) quality label and produced in the Italian Piedmont region, using culture-dependent and -independent approaches and determine mycotoxin and penicillin content using QToF LC-MS.

92 **MATERIALS AND METHODS**

93 **Sausage manufacture and sampling**

94 Three independent productions were carried out in an Italian factory located in the geographical area of
95 Piedmont region (North-West of Italy) following the *Salami Piemonte* PGI quality label specifications. The
96 formulation used in the manufacturing included pork lean meat from the shoulder and fat from the belly, salt
97 (maximum 3%), pepper (maximum 0.4%), spices and aromatic plants crushed or infused with wine. The
98 batches were produced in February, March and May, 2018. During the first stage of the fermentation
99 process, temperature decreased from 21°C to 15°C and relative humidity from 88% to 70%. After the first
100 week of fermentation and until the end of the maturation process, temperature and relative humidity were set
101 between 12-14°C and 74-88 %, respectively.
102 Concerning sampling, a total of 45 spontaneously fermented sausages (produced without the use of bacterial
103 starter cultures in the meat) and 45 natural casing samples, from the three batches, were collected at 4, 8, 15,
104 30 and 50 maturation days. At each sampling time, pH was measured from the first ten-fold serial dilution,
105 and a_w measurements directly from the meat sample using a digital pH meter (micropH2001; Crison,
106 Barcelona, Spain) and a calibrated electric hygrometer (HygroLab; Rotronic, Bassersdorf, Switzerland)
107 according to the manufacturer's instructions, respectively.
108 PGI specifications for this product permit the use of commercial starter cultures including bacteria and fungi
109 for meat and casing inoculation. However, in the framework of this study, the salamis were only inoculated
110 on the casing surface with a commercial starter culture of *P. nalgiovense*. The uninoculated casing was not
111 analysed due to logistic problems.
112

113 **Culture-dependent analyses of meat and casing samples to assess fungal diversity**

114 *Enumeration and isolation*

115 From each sausage, 5 pieces (1 cm^2) of casing randomly removed under sterile conditions using a sterile
116 scalpel or 10 g of meat were resuspended into 9 ml of sterile Ringer solution and vortexed vigorously for 1
117 minute. Ten-fold serial dilutions were performed and 100 µL aliquots were plated in triplicate on malt
118 extract agar (MEA, Oxoid) supplemented with 0.05 g L^{-1} tetracycline (Sigma, Milan, Italy) (Greppi *et al*.

2015). Plates were incubated for 5 days at 25 °C for fungal enumerations and isolations. Counts were expressed as mean values (log_{10} colony-forming units CFU per cm^2 or g of sample) of three technical replicates ± standard deviation. Given the low abundances of fungi in meat (mean abundance $<10^3$ CFU/g) as compared to casing samples, culture-dependent identification was only performed on fungi collected from casing samples. Approximately 20 fungal isolates were randomly picked from each batch and sampling time and purified on MEA media. Then, yeast isolates were cryopreserved in 30% v v^{-1} glycerol at -80°C after growth in YPD broth (dextrose 2%, bacteriological peptone 1%, yeast extract 1%) for 24h at 25°C. For filamentous fungi isolates, mycelium plugs removed from agar plates were cryopreserved at -80°C in pure sterile glycerol. A total of 275 isolates from casing samples (including 132 molds and 143 yeasts) were collected and used for downstream analyses.

Identification of fungal isolates

Moulds were presumably identified and dereplicated based on macroscopic and microscopic features (Pitt and Hocking 2009; Samson *et al*. 2010) and a total of 39 filamentous fungi were then selected for molecular identifications as described below.

Yeast isolates were dereplicated and presumptively identified by using the Fourier transform infrared (FT-IR) spectroscopy using a high-throughput system comprising a spectrometer (Tensor 27, Bruker Optics, Champs sur Marne, France) coupled to a high-throughput module (HTS-XT, Bruker Optics). After cultivation on yeast extract glucose chloramphenicol agar medium (YGC, Merck 1.16000) for 24 h at 27 °C, a half loop suspension of cells was suspended in 100 µl of deionised sterile water. Then, 25 µl of this suspension were deposited onto a ZnSe carrier and dried for about 45 min at 40 °C. Spectra acquisition and processing were performed as previously described (Kümmerle Michael, Siegfried Scherer, Herbert Seiler 1998) and spectra were compared to the Technical University of Munich reference database comprising about 2500 FTIR reference spectra. Analyses were performed using three technical triplicates. Yeasts from each batch were grouped according to their spectrum using the OPUS software program (Bruker, France) and representative isolates from each cluster were selected for molecular identification (total of 40 isolates).

DNA was extracted from scraped colonies for yeasts or mycelial plugs for molds using the FastDNA SPIN Kit (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. DNA was then diluted to

50–100 ng μl^{-1} and kept at −20 °C until further analysis. For yeasts, the D1/D2 domain of the 26S rRNA gene was PCR-amplified using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GTCCGTGTTTCAAGACGG-3′) (Kurtzman and Robnett 1997). For filamentous fungi, the partial β-tubulin gene (presumptive *Penicillium* spp.), partial elongation factor α (EFα) (presumptive *Cladosporium* spp.) or internal transcribed spacer (ITS) region (other genera) were amplified using primers Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass and Donaldson 1995), EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) (Carbone and Kohn 1999) and ITS4 (5′-CCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al.* 1990), respectively. PCR amplifications were carried out in a FlexCycler thermocycler (Analityk Jena, Germany) and PCR amplicons were sequenced at the Eurofins sequencing platform (Nantes, France) using the same primer pair. After sequencing assembly into contigs using the DNA Baser software (Heracle software, Germany), sequences were compared with the GenBank database using the "Basic Local Alignment Search Tool" (BLAST) (https ://www.ncbi.nlm.nih.gov/BLAST) for taxonomic assignment.

Culture-independent analyses of meat and casing fungal communities using metabarcoding

DNA was extracted from meat and casing samples obtained at each sampling point after centrifugation at 13,000 g for 10 min of 1 ml of the first ten-fold serial dilution in sterile Ringer solution . After removing the supernatant, total DNA was extracted from pellets using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) following the manufacturer's instructions. One microliter of RNase (Illumina Inc. San Diego. CA) was added to digest RNA in the DNA samples, with an incubation of 1 h at 37 °C. DNA was quantified using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and DNA concentration standardized at 5 ng μL^{-1}. DNA was subjected to amplification of the D1 domain of the 26S rRNA gene using the same primers (LS2-MF 5'-GAGTCGAGTTGTTTGGGAAT-3' and NL4 5'-GGTCCGTGTTTCAAGACGG-3') and procedure as described previously (Mota-Gutierrez *et al.* 2019). Briefly PCR was carried out using a mixture prepared with 12.5µl of the 2X Kapa HiFi HotStart ReadyMix Taq (Roche, Milan, Italy), 1 µmol L^{-1} each primer, 2.5 µl of DNA template, and PCR-grade water. PCR were subject to the following conditions: thirty cycles of 30 s of denaturation (95 °C), 30 s of primer annealing (55 °C), and 30 s of primer elongation

175 (72 °C), followed by a final elongation step (72 °C) of 10 min. PCR templates were then purified and
176 processed following the Illumina metagenomic pipeline. Sequencing was performed in a MiSeq instrument
177 in a 2X250bp configuration. After sequencing, reads were analysed by using QIIME following the detailed
178 pipeline described by Mota-Gutierrez *et al*. (2019). The 26S rRNA gene database from the same authors was
179 used for taxonomic assignment and OTUs centroids (picked at 99% of similarity) were double checked on
180 BLAST suite tools.
181
182 **Statistical data analysis**

183 OTU tables were rarefied to the lowest number of sequence/sample for each dataset. R was used to calculate
184 alpha diversity index using the *vegan* function. Principal component analysis (PCA) was also used for the
185 cluster analysis according to OTU composition and categorical variables. A one-way ANOVA was used to
186 analyze the effect of ripening time on dependent variables (mycobiota) separately for each batch by using the
187 IBM SPSS® Statistics 25 software with Duncan's Multiple Range Test (MRT) post hoc test with a
188 significance level set to 0.05. The *psyc* function in R was used for correlation analysis of OTU data from
189 casing and meat samples by spearman's rank method and the results were visualized using the *corrplot*
190 function in R.
191
192 **Extrolite extraction, detection and quantification in casing samples**

193 Since mycotoxins mostly accumulate in salami casing (Parussolo *et al.* 2019b), only casing samples were
194 analyzed. Casing samples (0.4 g) from sausages matured for 15, 30 and 50 days were analysed after liquid-
195 solid extraction. The extrolite determinations were then carried out on these samples by Quadrupole Time-
196 of-Flight liquid chromatography coupled to mass spectrometry (QTOF LC-MS; 6530 Agilent, France). The
197 following 17 extrolites were targeted: patulin (PAT), penitrem A (PEN A), andrastin A (AND A),
198 mycophenolic acid (MPA), citrinin (CIT), ochratoxin A (OTA), cyclopiazonic acid (CPA), aflatoxin B1
199 (AFLB1), (Iso)-fumigaclavine A (ISO-FUMI A), sterigmatocystin (STERIG), griseofulvin (GRISEO),
200 meleagrin (MELEA), eremefortins A (EREM A) and B (EREM B), roquefortine C (ROQ C), citreoviridin

201 (CITREO) and penicillin G (PEN G). Compound characteristics used for their detection and quantification
202 by QTOF LC-MS are shown in Table S1.
203 For extrolite extraction, casing samples were vortexed for 2 min in 6 mL acetonitrile-methanol-water
204 (30/30/40, v/v/v) solution and maintained in the dark for 1 h, then sonicated for 30 min followed by addition
205 of 6 mL pure hexane. Samples were placed on a RotoFlex Plus tube rotator (Sigma, France) for 10 min at
206 room temperature and centrifuged at 7000 g for 10 min at 4 °C. After discarding the hexane layer, 2 mL of
207 the acetonitrile-methanol-water phase were collected and stored at -20 °C until QTOF LC-MS analysis.
208 Before analysis, 1 mL of the solution was filtered through a 0.45 µm PTFE membrane syringe 4-mm filter
209 (Phenomenex, Torrance, USA) into an amber vial.
210 For extrolite detection and quantification, 2µL extracts were injected and compounds separated using a
211 ZORBAX Extend-C18 column (2.1×50mm and 1.8 µm, 600 bar) maintained at 35 °C. The flow rate was set
212 to 0.300 mL min-1 using as mobile phase: solvent A (milli-Q water + 0.1% v v^{-1} LC-MS grade formic acid
213 (Carlo Erba Reagents, France) + 0.1% v v^{-1} ammonium formate (Thermo Fisher Scientific, Waltham, MA,
214 USA) and solvent B (100% LC-MS grade ACN). Solvent B was maintained at 10% for the first 3 min,
215 followed by a gradient of 10-100% of B for 45 min. Finally, solvent B was maintained at 100% for 5-min
216 post-time. The analyses were done by ionization in both ESI+ and ESI- modes (ESI: Electrospray
217 Ionization), retention time values (± 1 min) and the corresponding qualifier and quantifier ions were used for
218 extrolite detection and identification as described in Table S1. Limits of detection and limits of quantification
219 values are also given in Table S1. Extrolite recovery from both meat and casing samples were determined
220 and ranged from 74 to 96% expect for citrinin and sterigmatocystin from meat samples (56% and 63%,
221 respectively) (Table S1). Quantification was performed using matrix matched linear ranges by preparing the
222 standards in blank sausage casing extracts.
223
224 **In vitro extrolite production**
225 *In vitro* extrolite production of one selected isolate from each *Penicillium* species were determined on Yeast
226 Extract Sucrose (YES) agar medium (10 g L^{-1} yeast extract, 150 g L^{-1} sucrose, 0.5 g L^{-1} $MgSO_4*7H_2O$, 0.006
227 g L^{-1} $ZnSO_4*H_2O$, 0.005 g L^{-1} $CuSO_4*5H_2O$, 15 g L^{-1} agar) buffered at pH 4.0 with phosphate-citrate buffer.
228 After distribution of 2 mL of YES medium into 24-wells plate, 20 µL of a 10^6 spores mL^{-1} suspension

prepared as described previously (Gillot *et al*. 2017) were deposited into each well in triplicate and incubated for 7 days at 25°C.

For extrolite extraction, 2 g of culture, comprising mycelium and medium, were weighed and homogenized in 6 mL of acetonitrile containing 0.1% formic acid (v v^{-1}) with an Ultraturrax T25 digital (IKA, Heidelberg, Germany) for 1 min. Then, 7.5 ml of acetonitrile containing 0.1% formic acid (v v^{-1}) was added to reach a final volume of 12.5 mL. After vortexing for 30 s, samples were sonicated in a sonication water bath for 15 min followed by vortexing again for 30 s before centrifugation at 5,000 g for 10 min at 4 °C. Two mL of the ACN phase were collected and stored at -20 °C until QTOF LC-MS analysis as described above. Before analysis, 1 mL was filtered through a 0.45 µm PTFE membrane syringe 4-mm filter (Phenomenex, Torrance, USA) into an amber vial. The extracts were only analysed for the presence or absence of each extrolite and *P. nalgiovense* commercial starter culture was used as a control.

RESULTS

Culture-dependent analysis of casing mycobiota

During the course of fermentation, pH values decreased between day 0 to 15 (March batch) or day 30 (February and May batches) followed by a pH-increase up to day 50 (Table S2). a_w was stable between day 0 and 8 with a mean value of 0.95-0.96 depending on the studied batch and then decreased to 0.91 by the end of maturation (Table S2).

Fungal counts significantly increased during fermentation in all studied batches, reaching ~6 $\log_{10}$ CFU cm^{-2} after 4 days of ripening and increasing up to 7 $\log_{10}$ CFU cm^{-2} after 8 days (Figure 1). From day 8 to the end of fermentation, an increase up to 7.7 $\log_{10}$ CFU cm^{-2} was observed. Comparison of fungal growth in the three batches showed a similar trend for fungal counts in the March and May batches between days 4 and 15 while those from the February batch were significantly lower ($P<0.05$). However, after 30 days, fungal counts were similar for all batches ($P>0.05$). Noteworthy, yeast counts were generally higher than molds at the beginning of ripening (day 4 and 8), then a progressive increase in mold counts was observed (data not shown).

255 Concerning fungal diversity, a total of 14 species comprising 4 yeast and 10 mold species were identified
256 (Figure 1). Yeast species included *Kodamaea ohmeri*, *Debaryomyces hansenii*, *Yarrowia divulgata* and
257 *Candida zeylanoides*. *D. hansenii* was found in all samples and represented 60% of total yeast isolates while
258 *C. zeylanoides* and *K. ohmeri* were isolated in February and March or March and May batches, respectively.
259 *Y. divulgata* was only isolated in the March batch, the latter being the only one where all four yeast species
260 were found.
261 Concerning filamentous fungi, five *Penicillium* species were isolated, i.e., *Penicillium crustosum*, *P.*
262 *cvjetkovicii, P. glabrum, P. nalgiovense* and *P. nordicum. P. nalgiovense* was the most dominant species in
263 all samples representing over 70% of total collected mold isolates. *P. cvjetkovicii* and *P. nordicum* were only
264 isolated in the March batch. *P. glabrum* was isolated in the May batch while *P. crustosum* was isolated in
265 February and May batches. We also identified the following *Cladosporium* species, *i.e., C. allicinum*
266 (February and March batches), *C. aggregatocicatricatum* (March batch) and *C. pseudocladosporioides*
267 (March and May batches) which all were subdominant (<5 % of total collected mold isolates) regardless of
268 the studied batch or sampling point. Finally, two saprobe species, i.e., *Trametes versicolor* and
269 *Paraophiobolus arundinis,* were also sporadically isolated.
270
271 **Culture-independent analyses of meat and casing mycobiota**
272 *Mycobiota of casing samples during maturation*
273 After quality filtering, a total of 932782 reads were used, with an average value of 62185 reads sample^{-1}, and
274 a mean sequence length of 381 bp. Good's coverage was ~98% indicating sufficient coverage for all samples
275 (Table S3A). The OTU relative abundances (%) are given in Table S4A.
276 The Good's coverage estimator was around 98% for each sample, indicating that sequencing depth was
277 satisfactory. Significant differences in alpha-diversity index, i.e., number of observed species and Shannon
278 index (Figure S1) were found between batches, with the May batch showing significantly higher values than
279 the other two batches.
280 Thirteen taxa (9 yeast and 4 mold taxa), with a relative abundance above 0.5% in at least two samples, were
281 observed. *Candida sake* (relative abundances ranging from 1% to 70%) and *Penicillium* sp. (ranging from

20% to 90%) were found in all samples, while the other dominant species were *D. hansenii* (relative abundances ranging from 5% to 40%), *C. zeylanoides* (relative abundance ranging around 5%) and *Y. divulgata* (relative abundances ranging from 4% to 12%) (Figure 2). OTU related to *Penicillium* sp. could not be identified to the species level.

PCA based on OTU table showed a sample grouping according to maturation time ($P < 0.05$) (Figure 3A) but not according to the studied batch while β-diversity analysis based on Bray-Curtis distances showed that both the batch of origin (Adonis test, $P < 0.05$, $r^2=0.1316$) and maturation time (Adonis test, $P < 0.05$, $r^2=0.2726$) shaped the casing mycobiota structure (Figure 3B). Indeed, in the PCoA, all samples collected in the February batch were grouped together while samples collected during early maturation (4 and 8 days) in March and May batches were grouped according to maturation time and those collected after 15 days were grouped according to their batch of origin (Figure 3B). Moreover, mycobiota structure of 15, 30 and 50d samples from the February batch were quite similar to those of the May batch (Figure 3B). Comparison of taxa relative abundances between batches showed that *D. hansenii* and *D. suglobosus* were only associated to February and May batches while *Y. divulgata* was only observed in the March batch (Figure S2). In addition, *C. zeylanoides* was significantly more abundant in the February batch than in the other batches. When comparing relative abundance values according to maturation time, we could observe that *C. sake* was significantly more abundant at early maturation stages (4 and 8 days) as compared to later maturation stages (Figure S3).

Mycobiota of meat samples during maturation

The total number of sequences obtained from meat samples throughout maturation was 805 054 raw reads. After quality filtering, a total of 72 9421 reads were used, with an average value of 48 661 reads/sample, and a mean sequence length of 392 bp. Good's coverage was ~98% indicating sufficient coverage for all samples (Table S3B). When comparing alpha-diversity index according to maturation time and batch of origin, no significant differences were observed for meat samples. The OTU relative abundances (%) are given in Table S4B.

As compared to casing samples, higher fungal diversity was encountered in meat than casings with 14 and 22 yeast and mold species found in meat samples, respectively. The core mycobiota of meat samples was

characterized by the presence of *Penicillium* sp. (relative abundance ranging from 10% to 80%), *Cladosporium* sp. (ranging from 1% to 7%) and *D. hansenii* (ranging from 1% to 25%) (Figure 4).

Even though PCA did not reveal any significant sample grouping according to time or batch production date (data not shown), significant differences in species relative abundances were observed between batches or according to maturation time. Indeed, the following yeast species were only observed in the May batch, *i.e., Candida tropicalis, Kluyveromyces marxianus* (relative abundances of ~2% on day 4, 15 and 30) and *Meyerozyma glycosophia* (relative abundances of ~3% on day 4 and of ~2% on day 8 and 30) (Figure S4). In addition, significantly higher relative abundances of *C. zeylanoides* and *Pichia occidentalis* (relative abundance ranging from 1% to 8%) were found in samples from February (Figure S4). In particular, a high relative abundance of *C. zeylanoides* was observed at the beginning of fermentation (day 4) in the February batch which then decreased during the maturation period (Figure 4).

Comparison of casing and meat mycobiota

Figure S5 shows the species for which significant differences (FDR<0.05) in relative abundances were found between the two sample types. Most of these species (21 out of 30 species) were only found in the meat samples and were not detected in casing samples. Except for *Aspergillus* spp., *Cladosporium cladosporioides*, and *Penicillium citrinum*, all these species had relative abundances below 1%. In contrast, *C. sake, Debaryomyces* spp. (*D. hansenii* and *D. suglobosus*) and *Penicillium* spp. were present in both sample types but were significantly more abundant in casing samples.

A correlation analysis of OTU data from casing and meat samples was performed using the spearman's rank method. As highlighted in Figure 5, presence of *C. zeylanoides*, *D. hansenii*, *S. cerevisiae* and *Pichia occidentalis* in the meat was positively correlated with the presence of *D. hansenii* and *C. zeylanoides* on casings while co-excluding pattern was shown between *Aspergillus*, *C. tropicalis*, *K. marxianus* and *M. glucosophila* in meat and *Y. deformans* and *Y. divulgata* in casing mycobiota.

Extrolite detection in situ and in vitro

Salami casing samples were screened for 17 different extrolites, including mycotoxins and penicillin G. All targeted mycotoxins were below their respective detection limits except for roquefortine C which was quantified at relatively low levels in March (22.86 ± 15.83 ng g^{-1} at day 15 and 21.62 ± 19.91 ng g^{-1} at day 30) and May (28.03 ± 25.25 ng g^{-1} at day 15, 46.06 ± 26.59 ng g^{-1} at day 30 and 47.99 ± 23.61 ng g^{-1} at day 50) batches (Figure 6). Penicillin G was not detected in any samples. Since only trace amounts of roquefortine C were detected in the casings, the meat samples were not analysed.

Isolates belonging to *P. crustosum, P. nalgiovense, P. cvjetkovicii, P. nordicum* and *P. glabrum* were all screened for their ability to produce mycotoxins and Penicillin G *in vitro*. Only *P. crustosum* was confirmed to simultaneously produce roquefortine C and penitrem A although penitrem A was not detected in casing samples. All other tested strains did not produce any of the target extrolites *in vitro* after 7 days growth on YES agar medium including the *P. nalgiovense* commercial starter culture that was used for inoculating the casing surface.

DISCUSSION

In the present study, an integrative approach combining culture-dependent and metataxonomic analyses was applied to decipher *Salame Piemonte* mycobiota. Mycobiota of traditional dry-cured meat products is usually characterized by the presence of mold species belonging to the *Penicillium* genus, mainly *P. solitum, P. nalgiovense, P. chrysogenum, P. olsonii, P. commune* and *P. salamii* (Perrone *et al*. 2015; Álvarez *et al*. 2020; Belleggia *et al*. 2020b; Iacumin *et al*. 2020; Zadravec *et al*. 2020). In the present study, *P. nalgiovense* was the most dominant mold species in all batches. It is a well-known technological mold used for dry-cured sausage production due to its positive influence on the final sensory characteristics of the product. The high dominance of *P. nalgiovense* throughout maturation is not surprising as *Salame Piemonte* casings were deliberately inoculated with a *P. nalgiovense* commercial starter culture. Moreover, because of its intense use in the factory and its presence on sausages during ripening, it is most likely present at high abundances in the air, e.g. drying and ripening rooms, which then act as an environmental source. It should be noted that there were major differences in *P. nalgiovense/Penicillium* spp. relative abundances depending on the approach

used. Indeed, this taxon was found at a high relative abundance over maturation time including early maturation using metabarcoding while it was found at a lower abundance using the culture-dependent approach. Such difference was particularly remarkable at the early stages of the February batch. A hypothesis explaining this difference is the fact that for filamentous fungi, viable counts usually reflect spore numbers rather than mycelium (Taniwaki *et al.* 2006). Indeed, it is likely that *P. nalgiovense* growth occured mostly as hyphae at early maturation stages thus allowing its earlier detection and at higher relative abundances with metabarcoding during maturation.

Besides *P. nalgiovense,* other mold species, probably transferred to the casing surface through airborne contamination or through the use of spices or salt, included other *Penicillium* spp., e.g. *P. crustosum, P. cvjetkovicii, P. glabrum, P. nordicum* and *P. oxalicum*, as well as *Cladosporium* spp. and *Scopulariopsis brevicaulis.* These species were identified at different sampling times in the different batches but were not part of the dominant mycobiota of casings reflecting proper management of air quality within the factory and the hurdle effect of the casing microbiota itself. This is especially true for *P. nalgiovense* that is often used as a protective culture against mycotoxin-producing molds in dry fermented sausages (Bernáldez *et al*. 2013). While certain mold species may contribute to the organoleptic properties of *Salame Piemonte*, as reported for *C. cladosporioides* that was considered as a beneficial fungal species in Portuguese and South American sausages (Parussolo *et al*. 2019a; Belleggia *et al*. 2020a), other species are known mycotoxin producers and should be monitored.

In the present study, we were also able to unravel yeast diversity and dynamics on the casing surface and highlight the dominance of *D. hansenii* in all studied batches. This species is also suggested to positively contribute to the organoleptic properties of fermented meat (Andrade *et al*. 2010; Perea-Sanz *et al*. 2020). Variations from batch to batch in yeast species abundances were observed, in particular for *D. hansenii* which was less abundant in the March batch for example, while *Y. divulgata* was only identified in the latter batch, and *C. zeylanoides* was more prevalent in the February batch. Such variations have been previously reported in other traditional fermented foods of animal origin (Viljoen *et al*. 2003; Belleggia *et al*. 2020a) and are not surprising given the fact that the yeast mycobiota originates from the raw materials and the factory environment. It is worth mentioning that several species found in the present study have been rarely reported in fermented meats, such as *Y. divulgata, Trametes versicolor* and *C. sake.* The latter two species

have been previously found in other dry cured meat products such as Italian San Daniele ham (Comi and Iacumin 2013) and Italian fermented sausages (Rantsiou *et al*. 2005). Noteworthy, *C. sake* was not isolated by our culture-dependent approach but metataxonomic analysis revealed its presence in all batches. This may be due to its slow growth and/or susceptibility to tetracycline which was used as a selective agent against bacteria for yeast and mold isolation. Indeed, a tetracycline minimum inhibitory concentration of 250-500 µg ml^{-1} was reported for clinical strains of *Candida* spp. (Blanco *et al*. 2017), which is similar to the one used in the isolation medium *i.e.*, 500 µg ml^{-1}. Further work would be necessary to confirm this hypothesis.

Y. divulgata was mainly detected in the March batch and its presence is quite unusual in this type of product in contrast to other *Yarrowia* spp., *i.e*., *Y. deformans* and *Y. lipolytica* which have been previously reported in salami products (Aquilanti *et al*. 2007; Belleggia *et al*. 2020b). The latter species is a widely reported as a spoilage agent of dairy and meat products but, at the same time, it can exert beneficial effects during the ripening of various cheeses and meat products and contributes to their organoleptic properties by aroma compound production (Nagy *et al*. 2013; Péter and Nagy 2019). *Y. divulgata,* which was originally described and isolated from meat products (Nagy *et al*. 2013), may have an undesirable effect on the product as this species is capable of producing high amounts of sweetener molecules, i.e., erythritol and mannitol from glycerol (Rakicka *et al*. 2016; Péter and Nagy 2019) with production rates higher than that of *Y. lipolytica* (Rakicka *et al*. 2016). It also possess high lipolytic activities (Nagy *et al*. 2019). It would be of interest to investigate further whether *Y. divulgata* could have a positive or negative impact on fermented meat sensorial properties.

Concerning the meat mycobiota, much higher species richness was found in meat samples throughout maturation as compared to casing samples and, in contrast to the casing mycobiota, we did not observe any significant sample grouping according to maturation time or batches. Noteworthy, fungal populations in meat were low, <3 log10 CFU. g^{-1} as compared to those encountered in casings. The dominant species encountered on the casings from the different batches were also found in the meat, e.g., *D. hansenii*, *C. sake, C. zeylanoides, Y. divulgata* and *Penicillium* sp. although not in the same proportions. Taking into consideration the environment and the meat itself could be an important source of fungi during early maturation steps, it is also clear from these data that despite the large species richness encountered in meat, especially for yeasts, only a few species could effectively colonize the casing surface. For example, yeast

421 species such as *Hanseniaspora, Kazachstania*, *Malassezia* and *Pichia* spp. and mold species such as
422 *Aspergillus* spp. were never found on the casing despite their presence in the meat. To go further, we
423 conducted a correlation analysis between the casing and meat mycobiota using the spearman's rank
424 correlation test. Interestingly, strong positive correlations were identified between *C. zeylanoides* in the meat
425 mycobiota and *Debaryomyces* spp., including *D. hansenii*, in the casings. In contrast, strong negative
426 correlations were found between *K. marxianus* in the meat and *Yarrowia* spp. in the casings. To our best
427 knowledge, no publications related to the interactions, between the above mentioned species exist and future
428 work would be necessary to confirm these observations. The biological reasons for these correlations remain
429 unclear but they could be either direct (e.g., mutualism, competition, amensalism) or indirect, involving one
430 or more other members of the microbial community (e.g., indirect mutualism or amensalism, apparent
431 competition, habitat facilitation) (Moon *et al.* 2010). Finally, considering the high fungal species richness
432 inside the meat, it is not clear what their contribution would be, especially for yeasts, to the overall
433 organoleptic properties of the final product. However, it cannot be excluded even though their population is
434 limited that they participate to product typicity and the various organoleptic properties of the final product. It
435 would therefore be of interest to investigate whether such species are metabolically active during maturation
436 using, for example, a metatranscriptomic approach.
437 Concerning safety aspects, different *Penicillium* species isolated in the present study could be considered as
438 hazardous due to their potential capacity to produce mycotoxins (Núñez *et al.* 2015). Indeed, several
439 mycotoxin producers, including *P. crustosum* (penitrem A and roquefortine C producer) and *P. nordicum*
440 (OTA producer) were isolated from the casings. Nonetheless, only trace amounts of roquefortine C were
441 detected in samples from the March and May batches, but not in samples of the February batch. This result
442 indicated the absence of a safety risk in relation to potential mycotoxins and penicillin G. In addition, except
443 for *P. crustosum* which produced penitrem A and roquefortine C *in vitro,* none of tested isolates, including
444 the *P. nalgiovense* starter culture, produced any of the targeted extrolites or penicillin G at levels above their
445 respective detection thresholds. Given the fact that *P. crustosum* (isolated in the May batch) was associated
446 to the *Salame Piemonte* mycobiota, we can hypothesize that these trace contaminations were due to this
447 species.

In the present study, mycobiota diversity and dynamics of a traditionally fermented meat product were investigated. This study highlighted the substantial fungal diversity in both meats and casing surface samples throughout maturation despite the fact that the product was deliberately inoculated with a *P. nalgiovense* commercial starter culture. To conclude, it could be of interest in the future to study the contribution of this mycobiota on the product volatilome profile using metabolomic analysis and metagenomics or metatranscriptomic approaches in order to clarify the important contribution of autochthonous fungi in the microbial ecology of this product.

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CONFLICT OF INTEREST

No conflict of interest declared

Research data for this article

Sequences are available at the NCBI Sequence Read Archive under the bioproject accession numbers PRJNA669122.

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604

AUTHOR CONTRIBUTIONS

LC, JM, KR and MC obtained the funding and supervised the study, edited and proofread the manuscript. LC, KR, JM, MC and IFr designed the experiments. IFr performed the experiments and analyzed all the data, drafted the manuscript. MRC provided technical assistance for DNA extractions for metabarcoding and for microbiological analysis and JLJ provided technical support with fungal identification. MC and EP performed LC-MS data acquisition and analyses. IFe provided technical support for metabarcoding analyses. All authors contributed to the article and approved the present version.

FIGURE LEGEND:

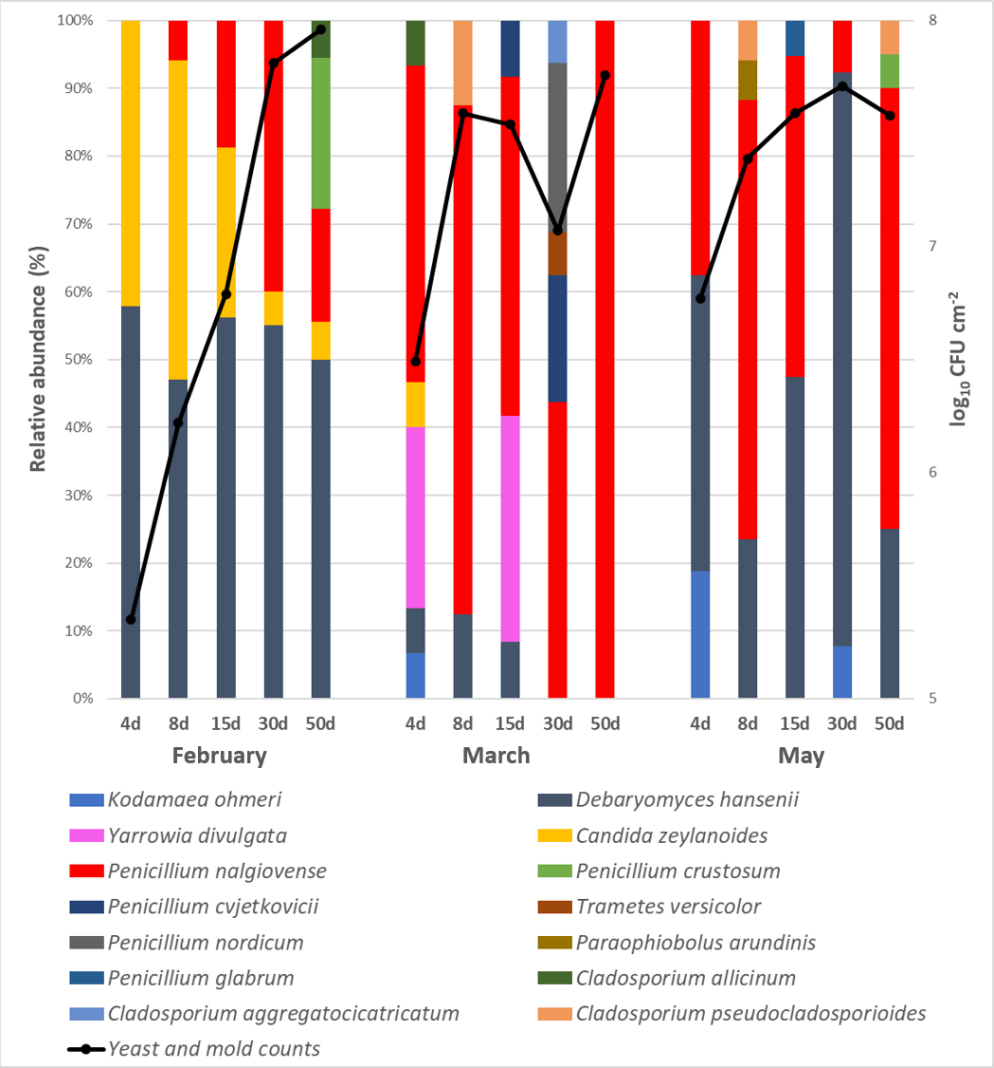
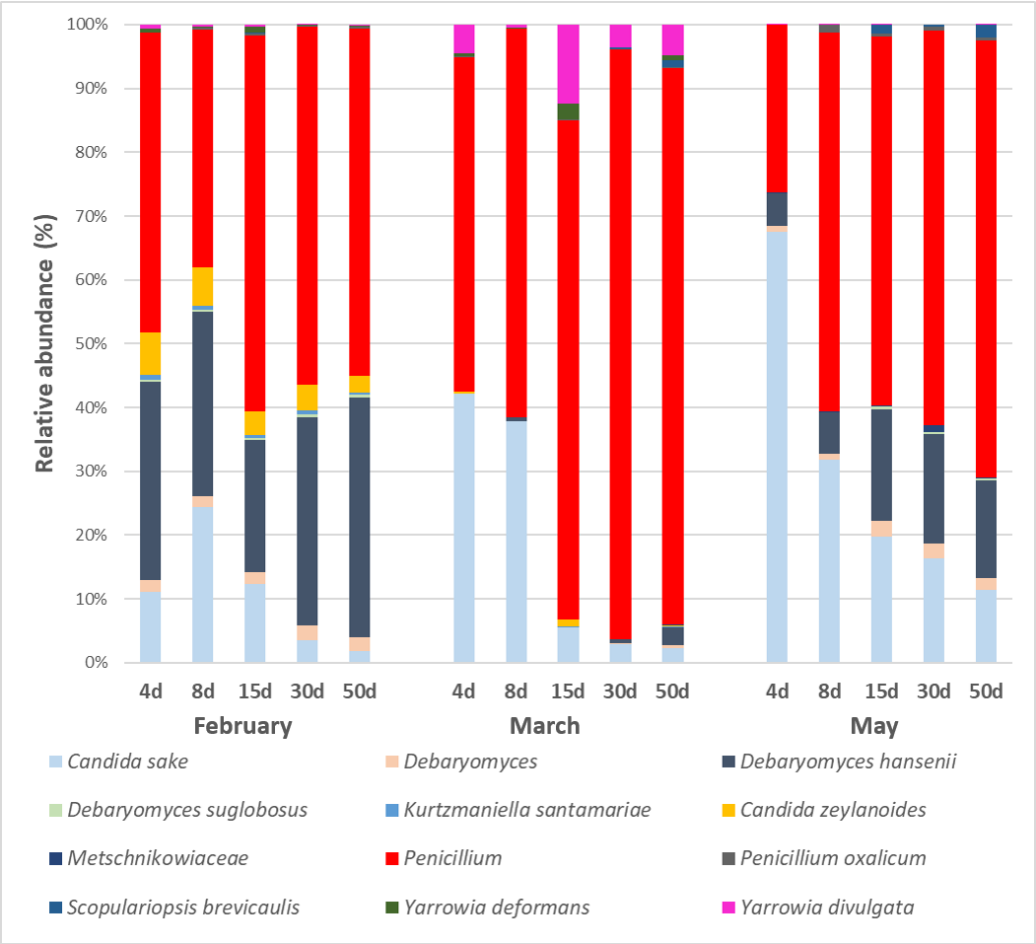


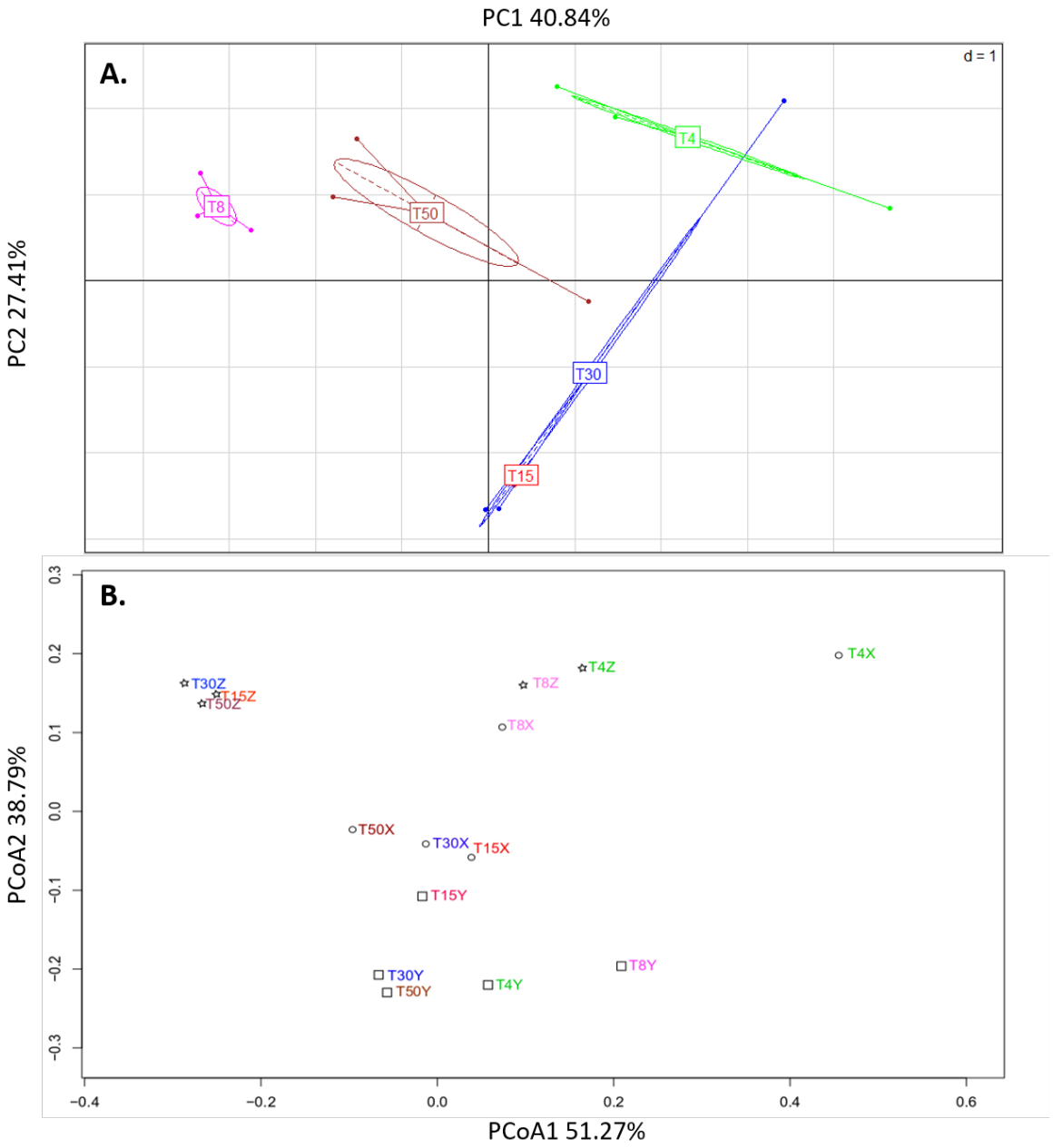
Figure 1. Fungal counts and taxa distribution in casing samples during different days (d) of fermentation of 3 Salame Piemonte batches using a culture-dependent approach.



618

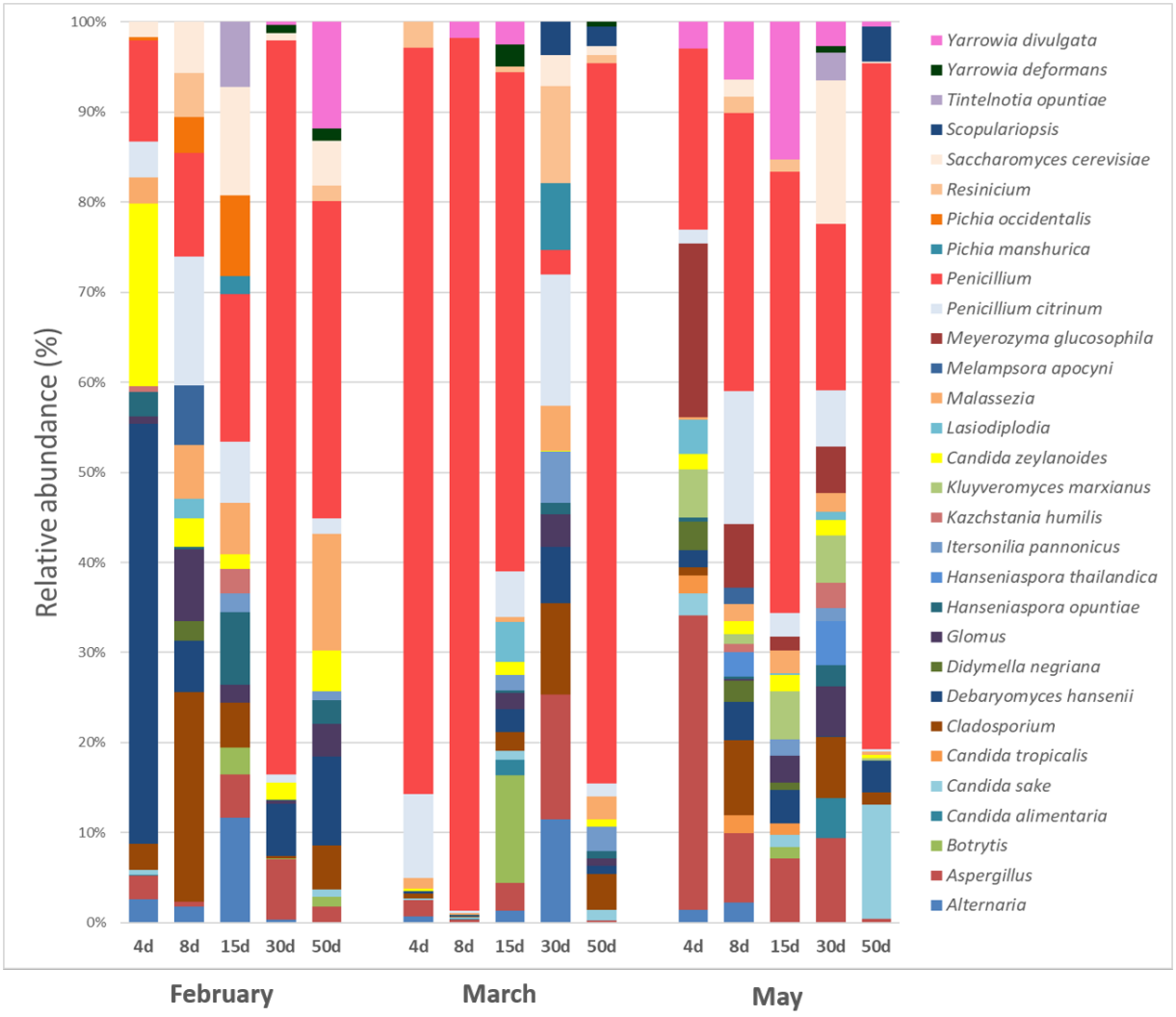
619 **Figure 2.** Taxa distribution in casing samples during ripening of three Salame Piemonte batches using a

620 metabarcoding approach. Only taxa with relative abundances above 0.5% in at least 2 samples are shown.



621

622 **Figure 3.** (A) Principal component analysis (PCA) showing casing sample mycobiota grouping according to
623 maturation time (Green= 4d; Pink= 8d; Red= 15d; Blue = 30d; Brown = 50d) and (B) Principal coordinates
624 analysis (PCoA) with the Bray-Curtis index showing casing sample mycobiota grouping according to
625 maturation time and batches (Y= February; Z= March; X= May).



626

627 **Figure 4.** Taxa distribution in meat samples during maturation of 3 Salame Piemonte batches using a

628 metabarcoding approach. Only taxa with relative abundances above 0.5% in at least 2 samples are shown.

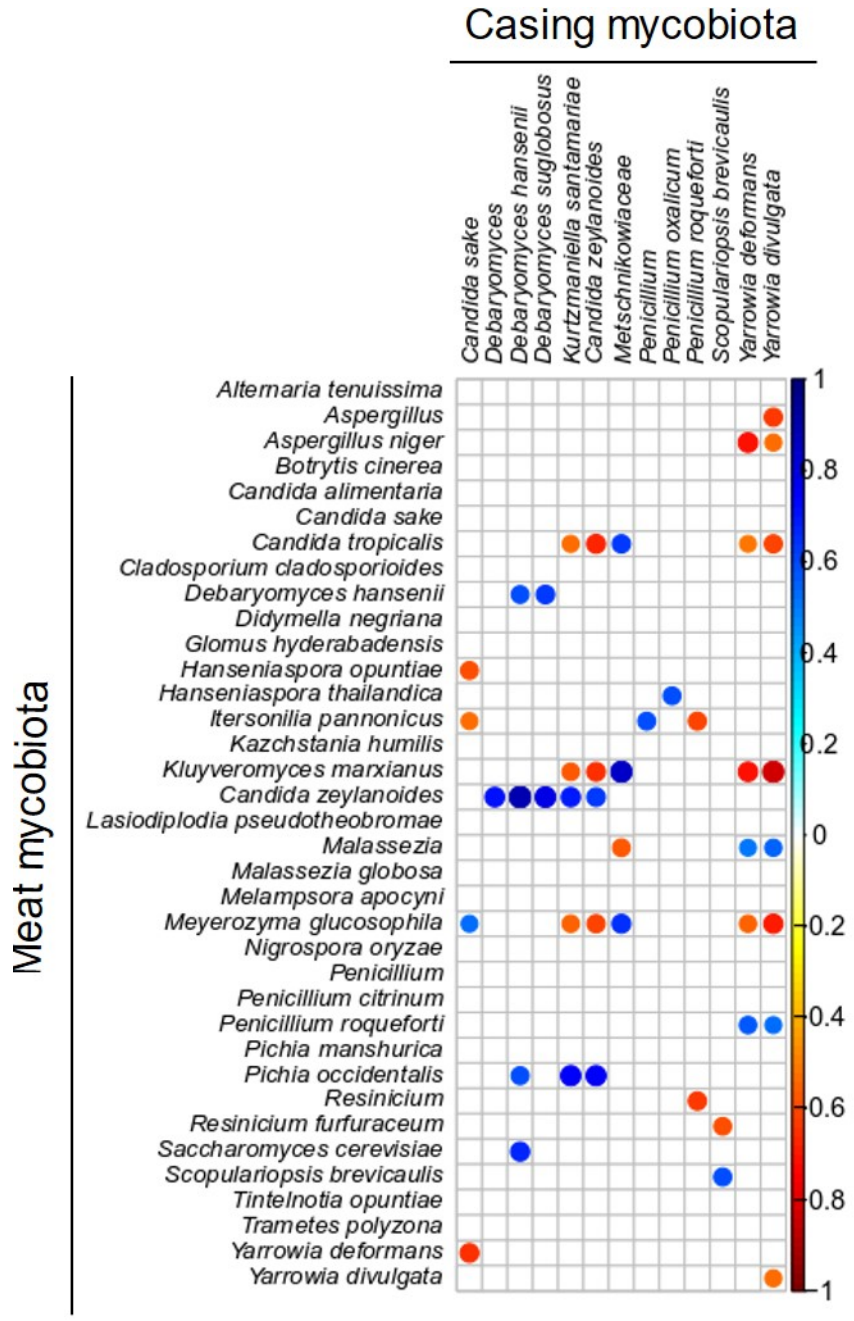
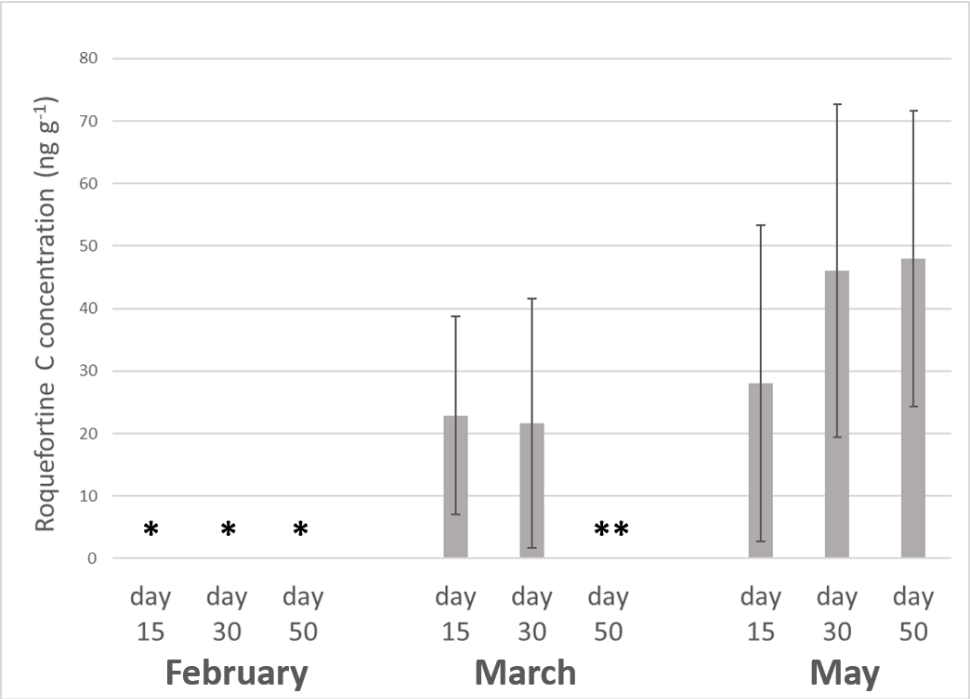


Figure 5: Spearman's correlation between the casing and meat mycobiota. (Only significant associations are shown, FDR-corrected $p < 0.05$). The colour intensity represents the degree of correlation where the blue colour represents a positive degree of correlation and red, a negative degree of correlation.



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634 **Figure 6:** Roquefortine C concentration (ng/g of casing) in samples with 15, 30 and 50 days of ripening for
635 each batch. *mean concentration below the limit of detection (< LOD); **mean concentration below the
636 limit of quantification (< QL).

637