Profiling of autochthonous microbiota and characterization of the dominant lactic acid bacteria occurring in fermented fish sausages

Profiling of autochthonous microbiota and characterization of the dominant lactic acid bacteria occurring in fermented fish sausages produced in Southern Italy

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Abstract

In the present study, culture-dependent and metaxonomic sequencing were applied to study the natural microbiota of fermented fish sausages handcrafted in Southern Italy. Sausages were also subjected to histamine quantification and real-time PCR for the quantification of the Gram-positive and Gram-negative *hdcA* and *hdc* genes, respectively, that codifies for histidine decarboxylase enzyme. Autochthonous lactic acid bacteria isolated from fermented fish sausages were subjected to PCR to detect *hdcA* gene, and of *epsA*, *epsB*, *epsD/E*, *epsEFG, gtf*, and *lev* genes, that codifies for the production of exopolysaccharides (EPS). Isolates were also screened for their ability to produce bacteriocins against *Listeria innocua*. In the analysed samples, high counts of presumptive lactic acid bacteria, coagulase-negative staphylococci, and eumycetes were detected. Moreover, Enterobacteriaceae,Pseudomonadaceae and enterococci were also detected. Regarding the microbiota composition, many bacteria and eumycetes commonly associated with fermented meat sausages were detected, thus suggesting their adaptation to a previously unexplored niche. Bacterial composition displayed the highest frequency of *Latilactobacillus sakei*, followed by *Clostridium*, *Latilactobacillus curvatus*, *Peptostreptococcus,* and *Bacteroides*. Eumycetic composition displayed the highest frequency of *Kurtzmaniella zeylanoides*, *Rhodotorula mucilaginosa*, *Debaryomyces hansenii*, *Galactomyces*, and *Galactomyces geotrichum*. Histamine content was highly variable among fish sausage samples, being comprised between < 4.2 mg Kg^{-1} and 299.6 ± 13.5 mg Kg^{-1}. The copy number of the *hdcA* gene of Gram-positive bacteria was below the detection limit in all the fermented fish sausage samples, whereas for the *hdc* gene of Gram-negative bacteria, values between 5.20 ± and 5.50 ± 0.26 Log cells/gene copies g^{-1} were detected only in one batch. In the present study, 60 lactic acid bacteria isolates (22 isolates of *L. sakei* and 38 of *L. curvauts*) from fish sausage samples were obtained. None of the *L. sakei* or *L. curvaus* isolates exerted a bactericidal effect against *L. innocua*. Among the isolates tested for the production of saccharose-dependent EPS, 39 out of 60 showed the formation of mucoid colonies. Interestigly, 56 out 60 isolates were positive for the gene *epsD/E*, whereas 37 out of 60 isolates were positive for gene *epsA*, all these genes codifying for the production of heteropolysaccharides. Of note, the EPS production capability showed by many lactic acid bacteria isolates could represent a starting point for their future selection as adjunct cultures to improve texture and sensory traits of the fermented fish sausages under study. No autochthonous strains were positive for the presence of the *hdcA* gene, thus suggesting their suitability for further testing as potential starter cultures. Further research is needed to understand microbial and physico-chemical changes occurring during fermentation.

Keywords: metataxonomic analysis; *Latilactobacillus sakei*; real-time PCR; histamine; *hdcA* gene

1. Introduction

The use of fermentation to preserve fish is one of the most ancient and effective methods to extend the shelf-life of this perishable food. Moreover, after fermentation, a range of flavorful taste compounds and aroma compounds are released in food matrix (Mouritsen et al., 2017), thus contributing to enhance the palatability of this product. The art of fish preservation dates back the Mesopotamians as early as the third millennium BC. Subsequently, the Ancient Romans were the major consumers of the so-called *garum*, a very famous fermented fish sauce imported from the East. Today, fermented fish preparations are very common in Asian countries as Japan, Thailand and Vietnam (Mouritsen et al., 2017; Zhao et al., 2019; Jang et al., 2017), Africa (Marti-Quijal et al., 2020), and in the northern Europe (Skåra et al., 2015). In Southern Italy, the so-called "*Colatura di Alici di Cetara*", which is a transparent liquid sauce with an amber color produced through ripening process of anchovies in a salt solution, represents a gastronomic excellence of undisputed value and attests the vocation of this Mediterranean area in the production of fermented fish products.
Usually, after fish harvesting, a rapid deterioration of flesh can occur due to the activity of autolytic enzymes and microorganisms such as *Pseudomonas*, *Vibrio*, *Photobacterium*, *Serratia*, *Shewanella*, *Aeromonas*, and those belonging to the family of Enterobacteriaceae, that naturally contaminate the fish gut and the environment (Zhou et al., 2021). To overcome this issue, fermentation process and eventually added ingredients allow spoilage microorganisms to be replaced by pro-technological microorganisms. Such a microbial succession leads to a safe preservation and a prolonged lifetime of the prepared foodstuff.
Among pro-technological microorganisms, lactic acid bacteria, naturally occurring in the raw material, or intentionally added as starter cultures, play a key role in driving fermentation, although many other microbial groups can be involved, depending on the process and the raw materials used. Lactic acid bacteria are a group of aero-tolerant Gram-positive bacilli or cocci, strictly fermentative, and organotrophic (Marti-Quijal et al., 2020). Homofermentative lactic acid bacteria produce lactate as the main final product of fermentation (Marti-Quijal et al., 2020; Zhou et al., 2021), whereas heterofermentative species metabolize both hexoses and pentoses leading to different metabolites, including CO_2, lactate and acetate or ethanol (Zotta et al., 2018). The microbial-derived organic acids lead to pH decrease with safety improvement and increase of the sensory characteristics of the product.
Besides the well-known acidifying activity, lactic acid bacteria exert many other biological activities that improve safety and rheological properties of the fermented product; among these activities, the production of bacteriocins and exopolysaccharides (EPS) is included.
By contrast, the metabolic activity of fermenting microorganisms can also exert adverse effects on the safety of foods by producing biogenic amines (e.g., histamine) that are precursors of carcinogenic N-nitroso compounds originating from the degradation of high-protein foods (Fong et al., 2021).
Hence, the success of the fermentation process is based on a fragile balance among the risks of cross-contamination, outcompeting wild fermentation, and occurrence of unwanted and potentially pathogenic fungal and bacterial species as well as toxic metabolites.
To the authors' knowledge, the use of fish flesh to produce fermented sausages is uncommon, especially in European countries where fermented sausages are mainly obtained using mammalian meat. Notwithstanding, in Southeast Asia, freshwater fish as *Ophicephalus micropeltes*, *Notopterus* spp., *Probarbus jullieni*, and *Priacanthus tayenus* are often used to produce fermented ground fish preparations (Khem et al., 2013). Moreover, Khem et al. (2013) developed a model fermented fish sausage from the marine species *Pseudocaranx dentex*, *Arripis trutta* and *Macruronus novaezelandiae*.
In the present study, different selective growth media and a metataxonomic approach were applied for the profiling of the microbiota naturally occurring in novel fermented fish sausages handcrafted in Southern Italy using marine fish species caught in the Mediterranean Sea. Sausages were also subjected to histamine quantification and a real-time PCR approach was used for the quantification of the Gram-positive and Gram-negative *hdcA* and *hdc* genes, respectively, in the food matrix. Moreover, in order to select lactic acid bacteria with potential pro-technological features, isolates obtained from the fermented fish sausages were subjected to PCR detection of the *hdcA* gene and of some of the genes involved in the production of EPS. The lactic acid bacteria isolates were also screened for their ability to produce EPS on appropriate solid media and bacteriocins against *Listeria innocua*.

2. Materials and methods

2.1. Sampling

Nine ready-to-eat fish sausages were collected from three production batches of an artisan producer located in Matino, Lecce, Italy. The fish sausage samples were codified as follows: FS1-FS3 obtained from batch 1 (expiration date 03.2021), FS4-FS6 obtained from batch 2 (expiration date 08.2021) and FS7-FS9 obtained from batch 3 (expiration date 01.2021). All samples were analysed before their expiration date. Each sample of ready-to-eat fish sausage (150 g weight) was produced with yellowfin tuna (*Thunnus albacares*) (50%), swordfish (*Xiphias gladius*) (30%), amberjack (*Seriola lalandi*) (15%), black pepper, garlic, cooked grape must, and mine salt. Fish sausages were stuffed into vegetable casings (not edible). Neither preservatives nor starter cultures were added. Prior to use, raw materials for the production of fish sausages have been subjected to a freezing treatment to prevent the risk of the parasite *Anisakis* sp. Defrosted fish were cut and trimmed to obtain fillets, that were subsequently sprinkled with salt, brown sugar and spices for a few days. Fish fillets were then cleaned of the excess marinade, minced and stuffed into vegetable casings. The fermentation was carried out in a seasoning cabinet at 20 °C and a relative humidity of 70-80% for 60 days.

2.2. Physico-chemical measurements

For pH determination, a pH meter equipped with a HI2031 solid electrode (Hanna Instruments, Padova, Italy) was used at the core of the fish sausages. The water activity (a_w) was measured by means of Aqualab 4TE apparatus (Meter Group, Pullman, USA) in accordance with ISO 21807:2004 standard method. To determinate the total titratable acidity (TTA), 10 g-aliquots were sampled from each fish sausage and homogenized with 90 mL of demineralised water in a Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy) at 260 rpm for 3 min. The TTA results were expressed as the total volume (mL) of a 0.1 N NaOH solution added to reach a fixed endpoint pH of 8.3. The D-/L-Lactic Acid (D-/L-Lactate) and Acetic Acid (ACS Manual Format) test kits (Megazyme, Bray, Ireland) were used to measure lactic acid and acetic acids contents, respectively, in accordance with the manufacturer's instructions. The analyses were performed in triplicate for each sample and the results were reported as mean ± standard deviation.
Histamine Assay Kit (Megazyme) was used to measure the histamine content of fish sausages. In more detail, 2 g of each sample were crushed by the use of a sharp knife and subsequently added with 15 mL of sample extraction buffer (100 mM EDTA, pH 8.0) placed into a heat resistant tube. Samples were then boiled for 20 min and left to cool at room temperature. After the further addition of sample extraction buffer in order to reach the total volume of 25 mL, sample was centrifuged at 10,000 rpm for 5 minutes. The supernatant was used for histamine quantification, as described by the manufacturer's instructions. Three biological replicates were analysed and the results were reported as mean of mg Kg^{-1} ± standard deviation.

2.3. Microbiological analyses

To perform microbiological viable counts, 10 g-aliquots of each fish sausage were homogenized with 90 mL of peptone water (Oxoid, Milan, Italy) (1 g/L) at 260 rpm for 3 min. The peptone water solution was used to carry out the serial ten-fold dilutions, in order to quantify the following microbial groups: (i) presumptive lactic acid bacteria, cultured on De Man, Rogosa and Sharpe (MRS) agar (VWR Prolabo Chemicals, Leuven, Belgium) (plus 250 mg/L of cycloheximide) with an incubation period of 48 h at 37 °C; (ii) coagulase negative staphylococci, cultured on Mannitol Salt Agar (MSA) (VWR Prolabo Chemicals) with an incubation period of 48-72 h at 37 °C; (iii) Enterobacteriaceae, cultured on Violet Red Bile Glucose Agar (VRBGA) (VWR Prolabo Chemicals) with an incubation period of 24 h at 37 °C; (iv) Pseudomonadaceae, cultured on Pseudomonas Agar Base (PAB) (VWR Prolabo Chemicals), plus cetrimide-fucidin-cephalosporin (CFC) selective supplement (VWR International, Milan, Italy), with an incubation period of 24-48 h at 30 °C; (v) enterococci, cultured on Enterococcus Selective Agar (Thermo Fisher Scientific, Buchs, Switzerland) with an incubation period of 48 h at 37 °C; (vi) eumycetes, cultured on Rose Bengal Chloramphenicol Agar (VWR Prolabo Chemicals) with an incubation period of 72-96 h at 25 °C. The analyses were performed in two biological and three technical replicates for each fish sausage and the results were expressed as the mean of Log of colony forming units (cfu) g^{-1} ± standard deviation.

2.4. DNA extraction and amplicon target sequencing

From the homogenates (dilution 10^{-1}) of each fish sausage sample, 1 mL-aliquots were obtained. The aliquots, containing about 100 mg of the respective sample, were centrifuged at 14,000 rpm for 10 min and the

supernatant was discarded. The pellets were processed for the total DNA extraction by using the E.Z.N.A. soil DNA kit (Omega Bio-tek, Norcross, GA, USA), in accordance with the manufacturer's instructions. To quantify the obtained DNA samples, the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) was used. The fish sausage DNA samples were then diluted to 5 ng/µL to carry out the amplicon target sequencing. In more detail, the bacterial biota was evaluated by the amplification of the V3-V4 region of the 16S rRNA gene by using primers and protocols described by Klindworth et al. (2013). Moreover, the D1-D2 domain of the 26S was amplified for the mycobiota evaluation, as reported by Mota-Gutierrez (2018). PCR amplicons were purified, tagged and pooled in equimolar concentration in accordance with the metagenomic standard procedure of Illumina. Pair-end sequencing (2X250bp) was performed with a MiSeq Illumina workstation (Illumina) with V2 chemistry following the manufacturer's instructions.

2.5. Bioinformatic analysis

Raw reads were imported in QIIME2 software (Bolyen et al., 2019) and primers and adapters were first trimmed by using Cutadapter and then quality filtered by using the DADA2 algorithm (Callahan et al., 2016). Low-quality bases and chimeric sequences were filtered out through the DADA2 denoise-paired plug of QIIME2 to obtain the Amplicon Sequence Variants (ASVs). The Greengenes 16S rRNA gene database was used for the taxonomic assignment of the bacterial biota, whereas the manually build database was used for the mycobiota (Mota-Gutierrez et al., 2018). The resulting taxonomic assignment of each ASVs was double-checked by Basic Local Alignment Search Tool (BLAST) performed manually. The sequencing data were deposited in the NCBI Sequence Read Archive (SRA) and are available under the Bioprojects Accession Number PRJNA769593

2.6. Detection of histidine decarboxylases-codifying genes in food matrix

DNAs obtained from the homogenates of fish sausage samples were subjected to qPCR in order to detect and quantify the *hdcA* gene of Gram-positive bacteria. To this end, a Mastercycler® ep realplex machine (Eppendorf, Hamburg, Germany) was employed by following the cycling program already described by Belleggia et al., (2021). The primer set Hdc1 (5'-TTGACCGTATCTCAGTGAGTCCAT-3') plus Hdc2 (5'-ACGGTCATACGAAACAATACCATC-3') was used to amplify the *hdcA* gene fragment (174 bp) (Fernández et al., 2006). The reaction mixes contained 4 µL of DNA sample, 5 µL of Type-it 2X HRM PCR Master Mix (QIAGEN), 900 nM of both primers and nuclease-free molecular biology grade water to reach the final volume of 10 µL. The positive strain *Lactobacillus parabuchneri* DSM 5987 was utilized to construct the standard curve. In detail, the strain was subcultured on MRS medium and suspended on sterile saline solution (0.85% NaCl, w/v) to perform 10-fold serially dilutions. The concentration of the undiluted suspension was calculated through the use of standard microbiological counting methods, including the Thomas cell counting chamber and plate viable counts on MRS agar. One mL of each dilution series point was subjected to DNA extraction, as described in paragraph 2.4. To create the standard curve, the Ct values of the qPCR performed on the extracted DNAs were plotted against the *hdcA* gene copies of each reaction. The efficiency (E) and the correlation coefficient (R^2) of the qPCR were automatically computed from the slope of the standard curve by the Mastercycler ep realplex v2.2 software (Eppendorf). For each qPCR reaction of the samples, the absolute number of gene copies was obtained on the base of the standard curve, ranging from ~ 10 to 10^6. Analyses were performed in technical triplicate, together with a blank. The results were expressed as the mean of Log gene copies or cells g^{-1} ± standard deviation, as it is reported that histamine-producing Gram-positive bacteria contain a unique copy of the *hdcA* gene (Satomi, 2016).
Likewise, the same DNA samples were subjected to qPCR for the detection of *hdc* gene of Gram-negative bacteria. To this end, the analysis protocol optimized by Bjornsdottir-Butler et al. (2011) was applied with some modifications. In detail, the primer set hdc forward (5'-TCHATYARYAACTGYGGTGACTG-3') plus hdc reverse (5'-CCRTTRGTNACRTAVCCCCA-3') was used to amplify the *hdc* gene fragment (139 bp) (Bjornsdottir-Butler et al., 2011). The reaction mixes contained 4 µL of DNA sample, 5 µL of Type-it 2X HRM PCR Master Mix (QIAGEN), 500 nM of both primers and nuclease-free molecular biology grade water to reach the final volume of 10 µL. The positive strain *Morganella morganii* DSM 30117 was subcultured on BHI agar (VWR Prolabo Chemicals) and utilized to construct the standard curve, as already described in this paragraph. For each qPCR reaction of the samples, the absolute number of gene copies was obtained on the base of the standard curve, ranging from ~ 10 to 10^6. Analyses were performed in technical triplicate, together

with a blank. The results were expressed as the mean of Log gene copies or cells g^{-1} ± standard deviation (Bjornsdottir-Butler et al., 2011).

2.7. Statistical analysis

To assess statistical differences within fish 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).
Alpha and beta diversity as well as permutational multivariate analysis of variance (ANOSIM) of the metataxonomic data were performed thought the *diversity* function of QIIME2. Microbiota as weel asmycobiota compositions were compared between the three batches by Wilcoxon rank sum test (P value adjustment method: FDR).

2.8. Characterization of the dominant lactic acid bacteria

2.8.1. Lactic acid bacteria isolation

As previously described, lactic acid bacteria from fish sausage samples were cultured in MRS agar added with cycloheximide (250 mg/L) and the resulting colonies were randomly selected and subsequently sub-cultured to purity in the same conditions. The obtained isolates were then stored at -80 °C.
Prior to further analysis, lactic acid bacteria have been thawed and sub-cultured twice on MRS agar at 37 °C for 48 h. DNA of lactic acid bacteria isolates was extracted according to Osimani et al. (2015) and its purity and quantity were verified with a NanoDrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). DNAs were standardized to a final concentration of 100 ng/µL 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 on 1.5% (w/v) agarose gel in 0.5X Tris/Borate/EDTA (TBE) buffer containing 0.5 µg/mL 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 of lactic acid bacteria isolates, represented in FASTA format, were analysed with UCHIME2 software tool to uncover chimeras (Edgar et al., 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 60 lactic acid bacteria strains were finally submitted to GenBank DNA database to acquire the respective accession numbers.

2.8.2. Molecular typing by REP-PCR of the lactic acid bacteria isolates

Lactic acid bacteria fingerprints were obtained by using Repetitive Extragenic Palindromic PCR (REP-PCR) with the (GTG)5 primer (5'-GTGGTGGTGGTGGTG- 3'). PCR mixture (final volume of 25 µL) contained 1 µL of template DNA (100 ng), 0.5 µL of (GTG)5 primer 1 µM, 0.5 µL of dNTPs (0.25 mM each), 1.5 µL $MgCl_2$ 25 mM, 2.5 µ L of 10X PCR buffer and 0.2 µL of a 5 U of DNA Polymerase (QIAGEN, Hilden, Germany). PCR conditions consisted of an initial cycle of 5 min at 95 °C followed by 30 amplification cycles (30 s at 90 °C, 1 min at 40 °C, 8 min at 65 °C) plus one final chain elongation cycle for 16 min at 68 °C. PCR products were resolved by electrophoresis on 2% (w/v) agarose gel in 0.5X TBE buffer at 120 V for 2 h. The REP-PCR profiles were imported in the BioNumerics v6.1 software (Applied Maths, Sint-Martens-Latem, Belgium). The dendrograms were calculated on the basis of the Dice coefficient of similarity, with the unweighted pair group method by using average linkages (UPGMA) clustering algorithm (Vauterin L. & Vauterin P., 1992). Principal Coordinates Analysis (PCoA) (*dudi.pco* function in *made4* package of R) was carried out on the fingerprint matrix generated through BioNumerics calculated on Euclidean distance (*vegdist* function in package *vegan* R).

2.8.3. Detection of hdcA gene in lactic acid bacteria isolates

The DNAs obtained from the 60 lactic acid bacteria strains isolated from fish sausage samples were tested for the presence of the *hdcA* gene. In this case, a PCR was conducted in My Cycler Thermal Cycler (BioRad Laboratories) with the following conditions: an initial step of 3 min at 95 °C, 40 amplification cycles (20 s at 95 °C, 30 s at 58 °C, 20 s at 72 °C) and a final elongation step of 10 min at 72 °C. Each PCR reaction consisted of 1 µL of template DNA (100 ng), 12.5 µL of MyFi™ Mix (Meridian Bioscience), 900 nM for both primers (Hdc1 plus Hdc2) and nuclease-free molecular biology grade water to reach the final volume of 25 µL. The results were checked by electrophoresis, the HyperLadder™ 100 bp (Meridian Bioscence) was used as molecular weight standard.

2.8.4. Antimicrobial activity of lactic acid bacteria isolates

The determination of the antimicrobial activity of the 60 lactic acid bacteria strains isolated from fish sausage samples was performed by following the Agar Well Diffusion Assay (AWDA), described by Parente et al. (1995). Briefly, molten BHI soft agar (0.75% agar) (VWR Prolabo Chemicals) was inoculated (2%) with the indicator microorganism *Listeria innocua*. Twenty mL of the inoculated medium were poured into 90 mm Petri dish and left to solidify. A cone of a 200 µL tip was utilized to create wells of ~ 50 µL capacity on BHI soft agar. Previously, lactic acid bacteria strains were sub-cultured twice on MRS broth at 37 °C for 48 h. The broth cultures were then added with 0.1 N NaOH solution to reach pH 7.0 in order to neutralise the organic acids produced during the bacterial growth. A filtration step on sterile PES membrane filter of 0.22 µm pore size (Laboindustria S.p.A., Padova, Italy) followed. For each lactic acid bacteria strain, 4 wells were formed on BHI soft agar, each containing: (i) 50 µL of the sub-cultured suspension; (ii) 50 µL of the neutralised suspension adjusted to pH 7.0; (iii) 50 µL of the filtered neutralised suspension; (iv) 50 µL of sterilised water as a negative control. Afterwards, the Petri dishes were incubated at 37 °C for 24 h and were examined for the presence of zones of inhibition. In the case of positive results (presence of inhibition halo), 3 spots of 5 µL each of pepsin (Fluka™, Honeywell, Morristown, USA), trypsin (Fluka™) or Pronase (Merck KGaA, Darmstadt, Germany) were set along the circumference of the inhibition zone to evaluate the protein nature of the antimicrobial. The Petri dishes were further incubated at the same conditions. The antimicrobial activity of lactic acid bacteria strains due to the synthesis of bacteriocins was confirmed by the formation of crescents.

2.8.5. EPS production of lactic acid bacteria isolates

The 60 lactic acid bacteria strains were screened for EPS production, based on the method already reported by Hilbig et al. (2019) with some modifications. In more detail, lactic acid bacteria isolates were retrieved from cryo-protective suspensions and sub-cultured twice on MRS broth at 37 °C for 48 h. The EPS production was visually observed by adding aliquots of 5 µL for each bacterial strain on the following 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 extract (10 g L^{-1}), galactose (20 g L^{-1}) and lactose (20 g L^{-1}) to promote the synthesis of heteropolysaccharides (HePS). After an incubation period of 48 h at 30 °C, the colonies were classified as positive whenever presenting a mucoid aspect (visible shiny and slimy appearance) or a ropy consistence (able to produce detectable filaments by using a sterile toothpick). The positive strains were additionally tested to confirm EPS production under stressed conditions on RSM agar, containing the following ingredients: meat extract (100 g L^{-1}), nitrite curing salt (0.5% w/w $NaNO_3$ in NaCl) (30 g L^{-1}), agar (15 g L^{-1}), tween 80 (1 g L^{-1}), sodium ascorbate (0.6 g L^{-1}) and lactic acid to reach a final pH of 5.8; moreover, sucrose (80 g L^{-1}) or glucose (20 g L^{-1}), galactose (20 g L^{-1}) and lactose (20 g L^{-1}) were added depending on the previous results. The positive phenotypes were confirmed after an incubation period of 120 h at 20 °C. For each strain, three spots were formed on the same plate and the analyses were conducted in duplicate.

2.8.6. Detection of EPS-related genes of lactic acid bacteria isolates

The presence of EPS-related genes on lactic acid bacteria strains was further investigated, as described by Milanović et al. (2020). In more detail, the EPS-related genes include *epsA*, *epsB*, *epsD/E* and *epsEFG*, involved in HePS biosynthesis, and *gtf* and *lev*, involved in HoPS biosynthesis. The extracted DNAs of lactic acid bacteria strains were amplified by PCR in My Cycler Thermal Cycler (BioRad Laboratories) following the conditions and primer pairs detailed by Palomba et al. (2012) and reported in the Supplementary Table 1. Each PCR reaction consisted of 3 µL of template DNA (300 ng), 25 µL of MyFi™ Mix (Meridian Bioscience),

1 μM of each primer and nuclease-free molecular biology grade water to reach the final volume of 50 μL. Few randomly selected amplicons testing positive after observation by electrophoresis were shipped to Genewiz (Takaley, UK) for their purification and sequencing to confirm the reaction specificity for each EPS-related gene. The resulting sequences were subjected to alignment with similar deposited in the GenBank database by BLAST search (http://www.ncbi.nlm.nih.gov/).

3. Results

3.1. Physico-chemical measurements

The results of the physico-chemical measurements of the fermented fish sausage samples are reported in Table 1.
pH values were comprised between 5.51 ± 0.01 (sample FS9) and 5.87 ± 0.04 (sample FS2) with no significant statistical differences among batches.
a_w values ranged between 0.92 ± 0.01 (sample FS9) and 0.94 ± 0.01 (samples FS4, FS5 and FS6). The statical analysis showed a significant difference between batches 2 and 3.
The results of the TTA were characterized by values between 21.8 ± 2.4 (sample FS4) and 27.8 ± 0.9 (FS8) mL of a 0.1 N NaOH solution. A higher mean value was reported for batch 2, whereas batches 1 and 3 showed the lowest mean values.
Regarding lactic acid content, high concentrations were generally detected in fish sausage samples. In more detail, lactic acid values of fish sausages were comprised between 1.336 ± 0.021 (sample FS1) and 2.368 ± 0.053 g 100 g^{-1} (sample FS9). The average values of batches 1 and 2 were statistically lower compared to batch 3.
The acetic acid was detected in all the samples, with lowest values comprised between 0.027 ± 0.001 (sample FS9) and 0.182 ± 0.003 g 100 g^{-1} (sample FS1). A significant lower mean was reported for batch 3, whereas batch 1 was characterised by the highest mean value.
Finally, the histamine content was highly variable among fish sausage samples; in fact, the values ranged from < 4.2 mg Kg^{-1} (sample FS8) to 299.6 ± 13.5 mg Kg^{-1} (sample FS1). The highest mean value was detected in samples from batch 1.

3.2. Microbiological analyses

The results of the microbiological viable counts of the fermented fish sausage samples are reported in Table 2.
Presumptive lactic acid bacteria showed the highest viable counts, varying from 7.57 ± 0.01 (sample FS9) to 9.12 ± 0.03 Log cfu g^{-1} (sample FS4). The statistical analysis showed significant differences among all the batches.
Regarding enterococci, counts were comprised from < 1.00 (samples of batch 3) and 4.96 ± 0.03 Log cfu g^{-1} (sample FS1). Batch 1 showed the highest mean value, whereas batch 3 the lowest.
As for coagulase-negative staphylococci, counts between 2.64 ± 0.06 (sample FS8) and 5.87 ± 0.04 (sample FS2) Log cfu g^{-1} were detected. The lowest average count was reported for batch 3, whereas batches 1 and 2 showed the highest average values.
The results of Enterobacteriaceae and Pseudomonadaceae viable counts showed the same trend; in fact, both microbial groups were < 1 Log cfu g^{-1} in batches 1 and 3, whereas Enterobacteriaceae and Pseudomonadaceae mean values of batch 2 were statistically higher, reaching up to 4.45 ± 0.29 (sample FS5) and 5.16 ± 0.05 Log cfu g^{-1} (sample FS5), respectively.
Finally, eumycete counts ranged between 4.24 ± 0.08 (sample FS5) and 5.59 ± 0.05 Log cfu g^{-1} (FS7). Batch 2 was characterized by the lowest average value, whereas those of batches 1 and 3 were statistically higher.

3.3. Microbiota composition

3.3.1. Bacterial composition

After sequencing and quality filtering, a total of 186,395 reads were used for the downstream analysis with an average of 10,355 sequence/sample and a sample coverage of 99%. The alpha diversity analysis showed the lowest level of complexity and a minor number of observations in samples belonging to batch 1, followed by batches 3 and 2 (Kruskal-Wallis $P < 0.001$). Bacterial composition displayed the highest frequency of

Latilactobacillus sakei (90, 69 and 79% in batch 1, 2 and 3, respectively), *Clostridium* (3, 9 and 6% in batch 1, 2 and 3, respectively), *Latilactobacillus curvatus* (1, 4 and 3% in batch 1, 2 and 3, respectively), *Peptostreptococcus* (1, 5 and 4% in batch 1, 2 and 3, respectively) and *Bacteroides* (0.5, 3 and 2% in batch 1, 2 and 3, respectively) (Fig. 1). In addition, several minor ASVs less than 1% of the relative frequency were shared between datasets. From the beta diversity calculation, based on the weight unifrac distance matrix (Fig. 2, panel A), a clear separation of the samples depending on the different batches was observed. The analysis of the frequency difference of the Amplicon Sequence Variants (ASVs) showed that *Latilactobacillus sakei* and *Staphylococcus succinus* were mainly associated with batch 1, *Turicibacter* with batch 3 and the other ASVs with batch 2 (FDR < 0.05).

3.3.2. Eumycetic composition

After sequencing and quality filtering, a total of 440,962 reads were used for the downstream analysis with an average of 24,498 sequence/sample and a sample coverage of 99%. The alpha diversity analysis showed the lowest level of complexity and a minor number of observations in samples belonging to batch 1, whereas no differences were observed between batches 2 and 3 (Kruskal-Wallis $P < 0.001$). Eumycetic composition displayed the highest frequency of *Kurtzmaniella zeylanoides* (54, 34 and 49% in batch 1, 2 and 3, respectively), *Rhodotorula mucilaginosa* (18, 46 and 29% in batch 1, 2 and 3, respectively), *Debaryomyces hansenii* (17, 4 and 11%), *Galactomyces* (5, 9 and 7% in batch 1, 2 and 3, respectively) and *Galactomyces geotrichum* (3, 5 and 4% in batch 1, 2 and 3, respectively). In addition, several minor ASVs less than 1% of the relative frequency were shared between all the datasets. By plotting the weight unifrac distance matrix (Fig. 2, panel B), no clear separation of the samples was observed. Moreover, *Debaryomyces hansenii* was the only ASVs discriminating the samples, as it was mainly associated with batch 3 (FDR < 0.05).

3.4. Detection of histidine decarboxylases-codifying genes in food matrix

The standard curve of the qPCR performed on the homogenates of fish sausage samples to detect the *hdcA* gene of Gram-positive bacteria showed an efficiency of 0.96 and a R^2 of 0.998. The detection limit was ~ 1 Log gene copies of cells per reaction. The results indicated that the copy number of *hdcA* gene was below the detection limit in all the fish sausage samples.
On the other hand, the standard curve of the qPCR performed to detect the *hdc* gene of Gram-negative bacteria showed an efficiency of XXX and a R^2 of XXX. The detection limit was ~ XXX Log gene copies of cells per reaction. Interestingly, the *hdc* gene was not detected in batches 1 and 3, whereas it was detected in batch 2. In more detail, samples from batch 2 showed *hdc* gene abundances of 5.20 ± 0.21 (sample FS4), 5.50 ± 0.26 (sample FS5) and 5.49 ± 0.18 Log cells/gene copies g^{-1} (sample FS6).

3.5. Characterization of the dominant LAB

The alignment results of 16S rRNA sequences obtained from the 60 lactic acid bacteria isolated from the fermented fish sausage under study are listed in Table 3. The BLAST search allowed to achieve an unambiguous identification for all the tested isolates. In detail, *L. curvatus* was the most frequently isolated species (38 isolates), followed by *L. sakei* (22 isolates). The lactic acid bacteria species were equally distributed among samples, apart for FS4 (batch 2) and FS9 (batch 3) isolates exclusively belonging to the species *L. curvatus*.
Lactic acid bacteria fingerprints obtained by REP-PCR were used to build a PCoA, as a function of the batch (Fig. 3). Each batch displayed the presence of similar *L. sakei* REP-biotype, whereas some strains belonging to batch 1 and 2 were from the same biotype (Fig. 3, panel a). Regarding *L. curvatus*, strains belonging to batch 2 were well separated from those isolated from batch 1 and 3 (Fig. 1 panel b), whereas strains belonging to batches 1 and 3 showed the highest degree of similarity.
Regarding the PCR to detect the presence of *hdcA* gene of Gram-positive bacteria on the 60 lactic acid bacteria isolates, the electrophoresis showed that no isolate was positive for the target gene.
The results of AWDA showed that none of the isolates exerted an inhibitory activity against *L. innocua*. In fact, the limited cases of zones of inhibition were attributed to the acidity of the sub-cultured suspensions, since the formation of crescents after the addition of pepsin, tripsin and Pronase did not occur.
The results of the screening of the 60 lactic acid bacteria isolates for the EPS production are reported in Table 3. In particular, 39 isolates showed sucrose dependent EPS production through the formation of mucoid

colonies. The EPS production, presumably HoPS, of such isolates was confirmed under stressed conditions on RSM agar. On the contrary, the sucrose independent EPS production was not registered in any case.
The screening of the isolates for the occurrence of EPS-related genes highlighted the presence of genes involved in HePS biosynthesis. In detail, 56 isolates harboured the gene *epsD/E*, involved in the glycosyltransferase synthesis, whereas 37 strains resulted positive for the presence of *epsA* gene, involved in EPS regulation. Such findings were confirmed by sequencing and alignment with similar deposited sequences in the GenBank database of randomly selected positive amplicons. The 60 lactic acid bacteria isolates showed the absence of the other EPS-related genes, including those involved in HoPS production.

4. Discussion

Italian tradition in charcuterie dates back to the 12th century, when many fermented meat products, mainly based on the use of swine meat, were developed. Based on traditional recipes and processes of fermented meat products, the fermented fish sausages investigated in the present study were produced. The obtained end-product represents a culinary uniqueness whose microbiota has never been investigated before.
Interestingly, the overall data collected on the analysed fish sausages highlighted many physico-chemical and microbiological traits that were more similar to those of fermented meat sausages rather than fermented seafood products.
As far as pH values are concerned, an overall homogeneity was observed among the analysed batches, thus attesting a high level of standardization of the process. The detected values were almost comparable with those reported by Li et al. (2021) in naturally fermented tilapia sausages that showed values comprised between 5 and 5.2 after 30 days fermentation. Conversely, pH values of the analysed samples were higher than those reported by Zhao et al. (2021) and Zhang et al. (2013) in naturally fermented tilapia sausages and fermented silver carp sausages, respectively, attesting at about 4.5 after 30 days fermentation. It is noteworthy that, in fermented foods, pH reduction represents one of the main hurdles towards spoilage and pathogenic bacteria, thus contributing to obtain a safe food. Generally, in meat sausages, a deacidification occurs at the end of fermentation, with pH increase at about 5.5; hence, it is likely that a similar process could also have occurred in the analysed fish sausages. More research is needed to investigate pH dynamics on the studied fish sausages for which it is recommended that pH lower than 4.4 is reached during fermentation.
Regarding a_w, the detected values were notably higher than those detected by Hu et al. (2008) in fermented silver carp sausages, which ranged between 0.86 and 0.82 after 48 days fermentation. A_w, coupled with pH, contributes to create a hostile environment for the multiplication of some unwanted microorganisms and therefore its monitoring is particularly useful.
The high values of lactic acid detected in the analysed samples attested the occurrence of a robust microbial metabolic activity from lactic acid bacteria with a prevalent homofermentative or facultative heterofermentative metabolism. Production of organic acids contributes to pH decrease and confers a pleasant taste to the end-product. High lactic acid concentration was also observed by Hu et al. (2008) in fermented silver carp sausages after 48 days fermentation.
In the analysed samples high loads of viable microorganisms were detected.
As for presumptive lactic acid bacteria, the counts were slightly lower than those detected by Zhang et al. (2013) in silver carp sausage that attested at above 10 Log cfu g^{-1}. In fish fermented sausages, lactic acid bacteria catabolize pentose or hexose during fermentation, leading to the production of organic acids, including lactic and acetic acid (Zhou et al., 2021). Moreover, fish muscle proteins are progressively hydrolysed during fermentation by both bacterial and fish proteinases (Udomsil et al., 2010). Among these enzymes, aminopeptidases convert peptides and/or oligopeptides to amino acids that further serve as a precursor for flavour formation (Udomsil et al., 2010). The high loads of lactic acid bacteria detected in the studied samples attest the high adaptation of such a microbial group to the fermented fish sausages, thus confirming their pivotal role in obtaining the end-product. Other studies reported that the lactic acid bacteria, especially *Pediococcus* and *Lactococcus*, play a key role in the definition of physico-chemical traits in naturally fermented fish sausages (Li et al., 2021; Zhao et al. 2021).
Regarding enterococci, found with variable loads in the analysed samples, species belonging to such microbial group have already been detected by Li et al. (2021) and Zhao et al. (2021) in naturally fermented tilapia sausages, thus attesting their adaptation to this specific food matrix. Zhang et al. (2013) reported that enterococci could be responsible of accumulation of tyramine in fermented fish sausages; interestingly, Zhao et al. (2021) recently discovered a significant contribution of enterococci in the accumulation of biogenic amines in naturally fermented tilapia sausages.

As for coagulase negative staphylococci, to the author's knowledge a lack of information is available in the scientific literature for further comparison of data. However, species of coagulase negative *Staphylococcus* have already been detected in different fermented fish preparations (Khusro et al., 2017). Interestingly, probiotic species of staphylococci were also isolated from fermented fish by Borah et al. (2016), thus suggesting the need for further in depth regarding this microbial group in the samples under study.
In the analysed samples, high counts of Enterobacteriaceae and Pseudomonadaceae were also detected. It is noteworthy that both these two families of bacteria encompass potential human pathogens or spoilage microorganisms that, in fermented foods, are inhibited by fermentation metabolites (e.g., organic acids, bacteriocins). Moreover, Enterobacteriaceae play a pivotal role in the formation of biogenic amines since it is known that *Escherichia coli* can adapt its metabolism by self-regulating low pH values and lysine-containing environments by decarboxylating lysine in order to generate cadaverine for neutralizing excessive H+ in the system (Meng et al., 2022). The presence of Pseudomonadaceae is not surprising since members belonging to this family have already been detected in fermented fish products as *jeotgal*, a Korean traditional fermented fish sauce, *myeolchijeot* (anchovy) (Singh et al., 2018), as well as in naturally fermented tilapia (Zhao et al., 2021) and silver carp sausages, in this latter with counts attesting at about 5 Log cfu/g after 48 h fermentation (Zhang et al., 2013). The presence of Enterobacteriaceae and Pseudomonadaceae in the fermented fish sausages suggests a possible lack of acidification during fermentation.
Regarding eumycetes, the counts detected in the analysed samples were in accordance with those reported by Zhang et al. (2013) in spontaneously fermented silver carp sausages and in *plaa-som*, a Thai fermented fish (Saithong et al., 2010). It can be hypothesized that, similarly to meat sausages, yeasts could exert the following effects: i) protective role against lipid oxidation; ii) allow proper drying by protecting the sausage against fluctuation in humidity; iii) release peptides, free amino acids and free fatty acids by lactic acid metabolization (Belleggia et al., 2020).
In order to obtain a more precise overview into the microbiota involved in the fermentation of the studied fermented fish sausages, a metataxonomic analysis, based on the study of microbial DNA, was coupled with the culture-dependent approach. In this context, amplicon sequencing represents by far the most used approach for metataxonomic studies (Parente et al., 2020).
In the present study, the metataxonomic approach allowed major and minor microbial species to be detected.
In more detail, among bacteria, *L. sakei* represented the dominant species at the end of fermentation in all the analysed samples. Although this pro-technological lactic acid bacterium is commonly detected in fermented meat sausages as one of the fermentation-driving species (Aquilanti et al., 2016), to the authors' knowledge, its occurrence in fermented fish sausages is uncommon. Interestingly, *L. sakei* has already been detected as major species in *rakfisk*, produced in Northern Europe from salmonid freshwater fish (Skåra et al., 2015), and as minor species in *jeotgal* (Jung et al., 2018). Moreover, *L. sakei* has been exploited as starter culture for the reduction of biogenic amines accumulation in *som-fug*, a Thai traditional fermented fish sausage (Kongkiattikajorn, 2015). The homolactic fermentation of hexoses, carried out by *L. sakei*, produces lactic acid via the glycolytic pathway, whereas acetic acid is produced through heterolactic fermentation of pentoses. Moreover, *L. sakei* produces proteinases and aminopeptidases which degrade muscle proteins, using free amino acids and nucleotides as source of energy (Belleggia et al., 2020b). *L. sakei* is also able to use glucose, fructose and different hexoses as primary energy sources (Ferrocino et al., 2018).
In the analysed samples, *L. curvatus* was detected as minority species. Also this lactic acid bacterium is one of the most detected in fermented meat sausages being able to hydrolyse muscle proteins (Casaburi et al., 2016; Sun et al., 2019). Interestingly, *L. curvatus* has recently been isolated from Korean traditional fermented seafood, showing potential cholesterol reduction effect (Kim et al., 2021). To the authors' knowledge, there is a lack of data regarding the presence of *L. curvatus* in fermented fish sausages.
It is noteworthy that both *L. sakei* and *L. curvatus* are able to produce EPS that could interact with food matrix and with other beneficial microorganisms. Indeed, it is known that EPS produced by lactic acid bacteria could improve taste, texture and stability of food products (Wang et al., 2019). Moreover, α-D-glucan and EPS containing glucose and mannose produced by *Lactobacillus* spp. showed significant prebiotic activity to increase the growth of probiotic bacteria (Wang et al., 2019). EPS produced by lactic acid bacteria could also exerts several health-promoting effects, such as anti-inflammatory, antioxidant and antibiofilm activities (Wang et al., 2019).
L. sakei and *L. curvatus* are also able to produce bacteriocins that could improve the safety of fermented foods, being active against foodborne human pathogens. Bacteriocins are bioactive antimicrobial peptides that are synthesized in the ribosome of numerous bacteria and released extracellularly (Zau et al., 2018). In more detail, sakacin produced by *L. sakei*, due to its antimicrobial effectiveness (e.g., against *Listeria monocytogenes*),

possesses a significant potential as biopreservative (Mapelli et al., 2019), whereas curvaticin 13, a bacteriocin produced by a *L. curvatus* strain, proved from long time to have a bactericidal effect against *L. monocytogenes* (Bouttefroy and Millière, 2000).
Based on these premises, the 60 isolates of lactic acid bacteria (22 isolates of *L. sakei* and 38 of *L. curvatus*) were subjected to *in vitro* characterization of some pro-technological features. In more detail, the production of bacteriocins (sakacin and curvaticin), the production of EPS and the detection of EPS-related genes were assayed with the future goal to obtain effective starter cultures to be used for process standardization. Indeed, as reported by Cruxen et al. (2019), the most promising microorganisms utilized as starter cultures are those isolated from native microbiota of artisanal/local products.
Isolates were also tested for the absence of the *hdcA* gene, that codifies for histidine decarboxylase enzyme and could potentially lead to histamine production in the food matrix.
Among the isolates tested for the production of saccharose-dependent EPS, 39 out of 60 showed the formation of mucoid colonies. Interestingly, Hilbig et al. (2019) reported that exopolysaccharide-producing lactic acid bacteria can positively affect spreadability, texture, and taste of fermented sausages. Hilbig et al. (2019) also suggested that the initial sugar content of the meat batter as well as the addition of different mixtures of saccharose and glucose should be evaluated in order to maximize the production of EPS. It is noteworthy that, as reported by Almansoory et al. (2020), pH, carbon and nitrogen sources, and inorganic ions strongly affect the production of EPS by lactic acid bacteria. Hence, although the isolates tested in the present study showed the presence of some of the genes involved in the production of heteropolysaccharides, not all the isolates were able to produce mucoid colonies on agar plates, being likely influenced by the above-mentioned parameters.
It is known that the production of bacteriocins is strain specific (Zau et al., 2018); for example, *Lacticaseibacillus paracasei* CNCM I5369 strain carries five genes (*orf010*, *orf012*, *orf023*, *orf030* and *orf038*) coding for class II bacteriocins (Belguesmia et al., 2020). Regarding the screening of isolates for the production of bacteriocins, it is likely that no isolate possessed the genetic determinants for bacteriocin synthesis, since no *L. sakei* or *L. curvaus* exerted a bactericidal effect against *L. innocua*, hence, data will not be further discussed.
Lactic acid bacteria isolates obtained in the present study were negative for the presence of the *hdcA* gene of Gram-positive bacteria; this finding agrees with the absence of the same gene in the food matrix and supports their potential use as starter cultures in driven fermentation processes.
It is noteworthy that occurrence of biogenic amines in fish-based foods represents one of the main health issues related to this specific fermented food (Belleggia et al., 2021). Among biogenic amines, histamine is produced by the degradation of the amino acid histidine via bacterial decarboxylation; in more detail, this reaction is determined by the activity of the enzyme histidine decarboxylase encoded by a gene cluster that includes the *hdcA* gene (Diaz et al., 2016). The *hdcA* gene products belong to the pyruvoyl-dependent histidine decarboxylases of Gram-positive bacteria, whereas Gram-negative bacteria use a different type of histidine decarboxylases that is pyridoxal phosphate dependent (Lucas et al., 2005).
In the analysed samples, variable levels of histamine were detected, thus suggesting the presence of microbial strains carrying histidine decarboxylases-codifying genes in the raw materials. In fact, the detected gene copies of the *hdc* gene of Gram-negative bacteria in batch 2 might be correlated with the histamine levels detected in the same batch. It is likely that the detected gene copies could have been carried by members of the Enterobacteriaceae family, however, further research is needed to better clarify the origin of the detected gene.
On the other hand, the histamine content detected in batches 1 and 3 remains to be clarified. Although the qPCR assays prove to be valid and rapid for a proper evaluation of histamine risk in fermented foods, the detection limit of ~ 4 Log cells/gene copies g^{-1} appears unsatisfactory for certain applications. Moreover, as reported by Bjornsdottir-Butler et al. (2011), the primers used in the present study for the *hdc* gene of Gram-negative bacteria are suitable to detect high-histamine producing bacteria but exclude low- or non-detectable histamine producing bacteria. Although the qPCR assays provide quick and sound results in cases of high detection limits, its integration with other methods, in order to avoid the risk of histamine formation and scombroid poisoning, could be suggested.
Among biogenic amines, histamine is mostly implicated in food poisonings with potential health risks for consumers. In order to protect the health of consumers, in the European Union, the Commission Regulation (EC) 2073/2005 lays down food safety criteria for histamine in fishery products obtained from fish species associated with a high amount of histidine, between 100 mg/Kg and 200 mg/Kg. The same Regulation also establishes limits of histamine between 200 mg/Kg and 400 mg/Kg for fishery products subjected to enzyme maturation treatment in brine. Moreover, according to the United States Food and Drug Administration (FDA) (2001), histamine levels of fishery products should not exceed 50 mg/Kg, whereas a value over 500 mg/Kg

represents a possible risk for the health consumers (Belleggia et al., 2021). In the present study, only samples from one production batch showed high values of histamine, although, according to Commission Regulation (EC) 2073/2005, fermented fish sausages do not fall into food categories that have to be monitored for histamine content. It is noteworthy that, according to the European Food Safety Authority (EFSA) BIOHAZ Panel (2011), the evaluation of histamine risk in foods requires a case-by-case approach, since each food type is characterized by specific formulations and process parameters. Of note that, in acidic fermented foods, although the decrease in pH is recognized as a crucial factor to reduce the multiplication of contaminating microbiota, such phenomenon can in turn promote decarboxylation reactions carried out by surviving microorganisms in response to acidic stress (Vidal-Carou et al. 2007).
Finally, metataxonomic analysis allowed major and minor eumycetes species to be identified, to the authors' knowledge, eumycetes in fermented fish products are less investigated and further comparison of data is difficult to be performed. In the present study *K. zeylanoides*, *R. mucilaginosa*, and *D. hansenii* constituted the dominant species. Interestingly, all these species are constantly detected in fermented meat sausages, thus suggesting their adaptation also to fermented fish sausages where they could presumably exert similar metabolic activities to those performed in meat-based matrices.
As for *K. zeylanoides* (formerly known to as *Candida zeylanoides*), this yeast has already been found in *Suan yu*, a Chinese traditional fermented fish (Zang et al., 2018), in traditional Icelandic fermented fish (Osimani et al., 2019), and in different fermented sausages produced in Southern Italy (Belleggia et al., 2020). Moreover, members belonging to *Candida* species were detected in ethnic dried and salted or fermented fish products of Nepal and Northeast India, as *bordia*, *gnuchi*, *lashim*, *hentak*, *karati*, *sidra*, and *ngari* (Thapa, 2016).
Regarding *R. mucilaginosa*, this yeast species is commonly part of the minor fraction of the mycobiota of fermented sausages (Belleggia et al., 2020). Interestingly, Li et al. (2010) isolated *R. mucilaginosa* as marine-derived yeast from surface of marine fish, thus suggesting the adaptation of such yeast to marine environment and explaining its presence in the fish flesh used for the production of the fermented sausages under study.
D. hansenii represents one of the key yeast species detected in the fungal community of fermented meat sausages (Belleggia et al., 2020). *D. hansenii* is a halotolerant, lipolytic yeast able to hydrolyze muscle proteins, thus contributing to the definition of the sensory traits of fermented sausages. Moreover, the metabolic activity of this foodborne yeast leads to the increase of ammonia concentration and the decrease of lactate and acetate content (Belleggia et al., 2020). Interestingly, *D. hansenii* was isolated from the gut of rainbow trout reared in fresh water (Reyes-Becerril et al., 2008), hence, it is likely that the presence of such yeast species in the fermented sausages under study could be ascribed to cross-contamination between the gut content and the flesh of fish during slaughtering.

5. Conclusion

The data overall collected allowed a first picture of the microbiota of novel fermented fish sausages to be drawn. Regarding the microbiota composition, many bacteria and eumycetes commonly associated with fermented meat sausages were detected, thus suggesting their adaptation to a previously unexplored niche where *L. sakei* was surprisingly found as dominant species in all the analysed batches. As for lactic acid bacteria, none of the isolates showed the presence of genes for the decarboxylation of histidine, thus suggesting their suitability for further testing as potential starter cultures. Interestingly, the EPS production capability showed by many lactic acid bacteria isolates could represent a starting point for their future selection as adjunct cultures to improve texture and sensory traits of the fermented fish sausages under study. Further research is needed to understand microbial and physico-chemical changes occurring during fermentation of fish sausages, and to verify the *in vivo* performance of the isolates in the fish flesh. A further in-depth is also recommended to shed a light into bacteria-related histamine production dynamics.

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FIGURE CAPTIONS

Fig. 1. Relative frequency of the bacterial ASVs detected by sequencing. Only ASVs with an incidence above 0.5% in at least two samples are shown.

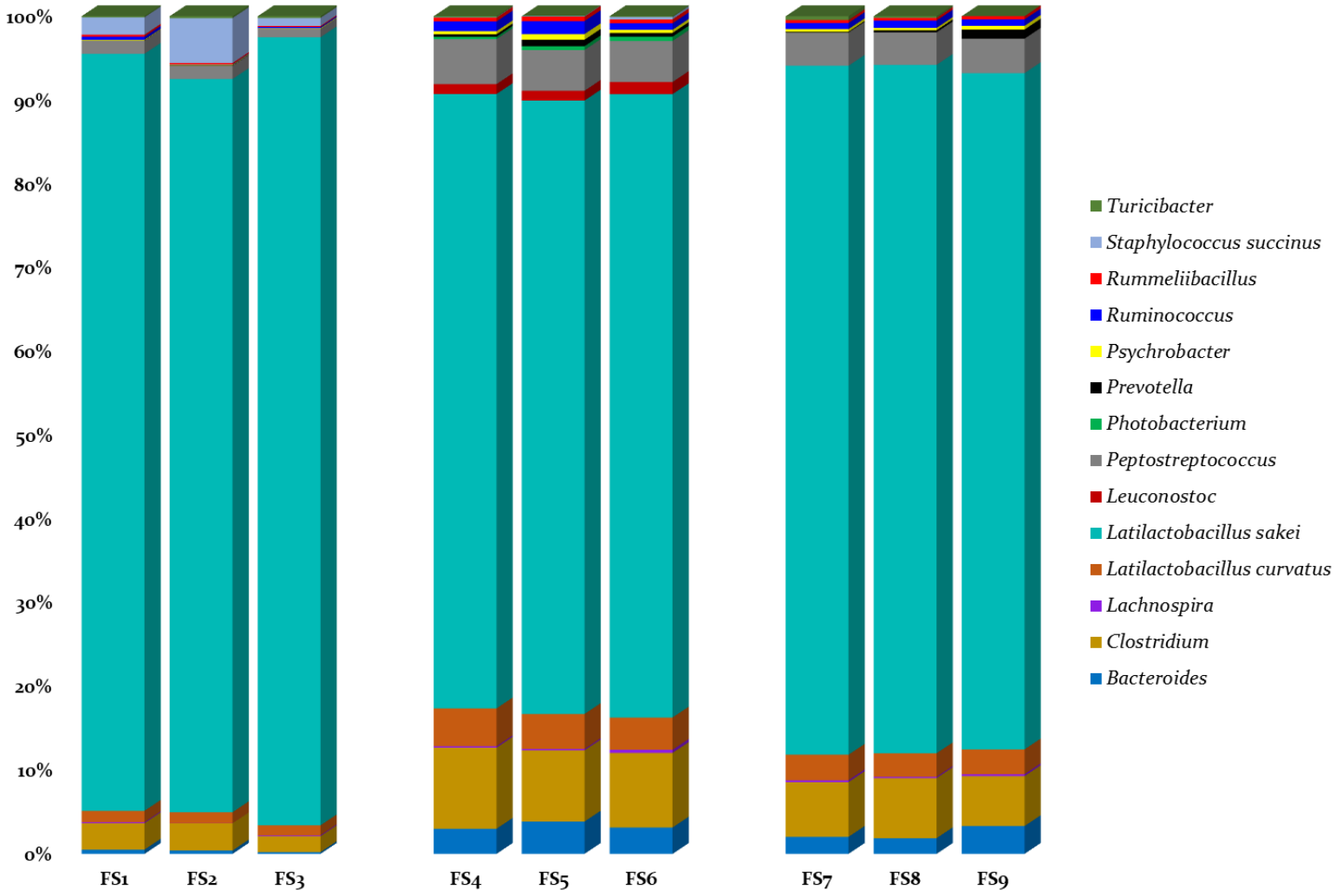


Fig. 2. Relative frequency of the fungal ASVs detected by sequencing. Only ASVs with an incidence above 0.5% in at least two samples are shown.

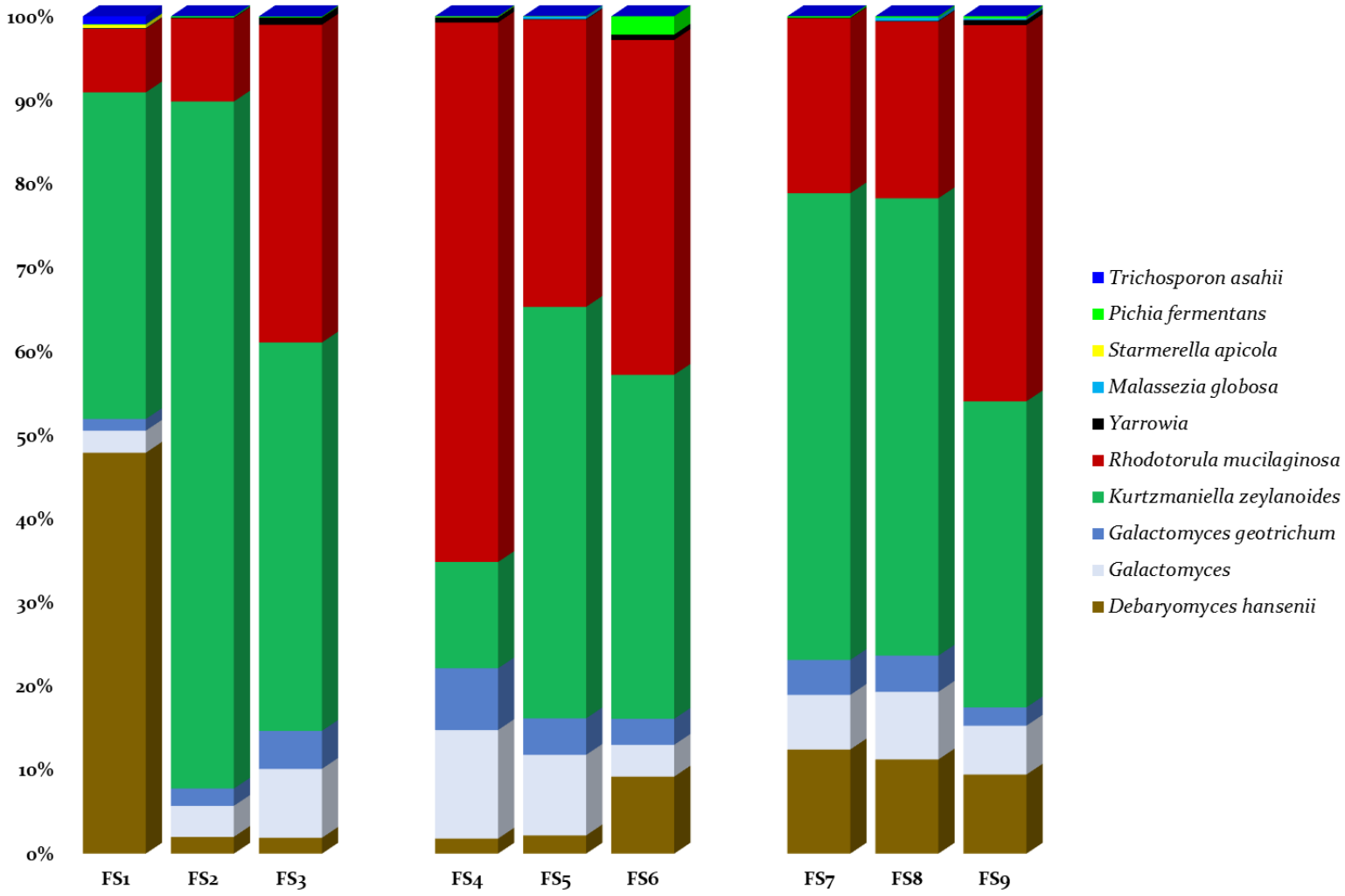
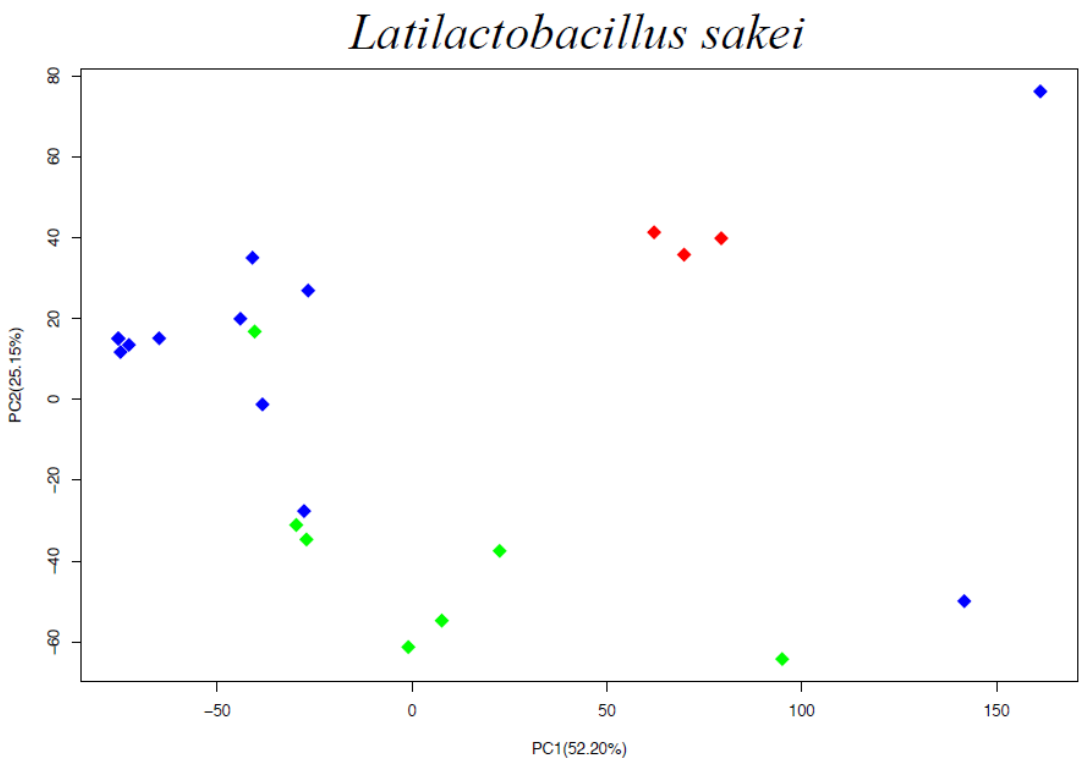


Fig. 3. Principal coordinates analysis based (PCoA) based on *L. sakei* and *L. curvatus* Rep fingerprints. The samples are color-coded according to the batch (A , blue; B, red; and C, green).

a)



b)