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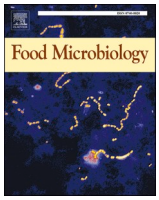

Characterization of dry-cured ham microbiota at 12 months of seasoning obtained from different rearing strategies using 16S rRNA profiling

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ABSTRACT

In this study, we investigated the microbiota of 72 Italian ham samples collected after 12 months of seasoning. The hams were elaborated from pigs fed different rearing methods, including the traditional restricted medium protein diet chosen as control (C group); restrictive low protein diet (LP group); two *ad libitum* high-protein diet groups (HP9M group: slaughter at 9 months of age; HP170 group: slaughter at 170 kg). A multi-amplicon 16S metabarcoding approach was used, and a total of 2845 Amplicon Sequence Variants were obtained from the 72 ham samples. Main phyla included: Firmicutes (90.8%), Actinobacteria (6.2%), Proteobacteria (2.7%), and Bacteroidota (0.12%). The most common genera were *Staphylococcus, Tetragenococcus, and Brevibacterium.* Shannon index for α-diversity was found statistically significant, notably for the HP9M group, indicating higher diversity compared to C. PERMANOVA test on β-diversity showed significant differences in rearing methods between HP170 and C, HP170 and LP, and HP9M vs. C. All three rearing methods revealed associations with characteristic communities: the HP9M group had the highest number of associations, many of which were due to spoilage bacteria, whereas the LP group had the highest number of seasoning-favourable genera.

1. Introduction

In the context of Italian pig production, dry-cured ham occupies a prominent position among high-quality products. The most recognized Protected Designation of Origin (PDO) production chains are Prosciutto di Parma (80,230 tonnes produced) and Prosciutto di San Daniele (26,603 tonnes produced), contributing to a total production and export value of € 983 and € 362 million, respectively (ISMEA Fondazione Qualivita, 2022). In addition to those, Prosciutto Crudo Veneto PDO represents a niche production, particularly appreciated in north-east Italy (843 tons per year for a production value of about €10 million; ISMEA Mercati, 2022). The thighs, with or without trotter, used for dry curing are obtained from pigs slaughtered at least at nine months of age with a weight of 160 ± 10%, as required by the main PDO product specification of the different consortia (Bosi and Russo, 2004). PDO production guidelines were recently revised by the consortia to meet the demands of the Italian market addressing the issue concerning the increase in fresh hams wastage at processing facilities due to their excessive leanness.

Recent studies have underpinned how a change in diet formulation and administration (*ad libitum* instead of a restricted regimen) can lead to benefits in terms of average daily gain and pig growth time while maintaining the chemical and physical characteristics required by the guidelines (Toscano et al., 2023).

The production process of dry-cured ham is characterized by a long curing and drying period of at least 18–20 months (pre-salting, salting, drying, and ageing), under controlled environmental conditions (air, light, relative humidity, pressure, and temperature). The combination of these two conditions is pivotal for the development and maintenance of physical and chemical properties such as visible fat, salt content, texture,

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and colour, as well its characteristic sensory traits (Bosi and Russo, 2004; Čandek-Potokar and Škrlep, 2012; Pagliarini et al., 2016). Furthermore, studies emphasized that factors such as rearing strategies, breed, age, slaughter weight, and the pigs handling conditions during slaughter concur as well in defining the fresh ham characteristics (Gallo et al., 2015; Schiavon et al., 2018).

During the production process, green hams undergo not only chemical and physical changes but also microbiological changes (Lin et al., 2020). The involvement of microorganisms in dry-cured ham seasoning is crucial for the development of the ham's typical flavour and aroma as reported by Hinrichsen & Pedersen (1995) and Wang et al. (2021). Studies on the microbiota of dry-cured hams exist either at the level of simple characterization (Cordero and Zumalacárregui, 2000; Martínez-Onandi et al., 2019; Woods et al., 2019) or in relation to stages of the production process (Mu et al., 2020) or flavour profile (Wang et al., 2021; Zhu et al., 2021). However, there are limited studies on the microbiota of Italian PDO dry-cured hams (Hinrichsen and Pedersen, 1995), although this product is one of Italy's most important production chains.

Given the relevance of the PDO dry-cured ham at the national and international level, the revision of consortia guidelines, and the need to comprehensively characterize the microbiota of hams under different rearing strategies, we explored, throughout sequencing of 16S rRNA, a specific PDO, Prosciutto Crudo Veneto, to define the microbiota of hams at 12 months of maturation under different feeding conditions (Toscano et al., 2023). The rearing strategies used are the traditional one, in which pigs are fed a restricted medium-protein diet chosen as a control (C), a restricted low-protein diet up to 170 kg body weight (LP), and two *ad libitum* high-protein feeds, one fed up to 9 months of age (HP9M) and one up to 170 kg body weight (HP170).

2. Material and methods

2.1. Animal data

The *in vivo* experiment and the composition of the diets are fully described in Malgwi et al. (2021).

Briefly, the Goland C21 pigs (n = 112) used in this study for the production of dry-cured ham were fed according to 4 different rearing strategies: (i) a traditional restricted diet with medium protein content, corresponding to the control strategy (C, 29 animals); (ii) a restricted diet with low protein content (LP, 28 animals), where the pigs were slaughtered at 170 kg of weight regardless of the months of age (>9 months); a high protein diet which was fed in two ways: (iii) *ad libitum* until they reached 9 months of age and a weight greater than 170 kg (HP9M, 28 animals); (iv) *ad libitum* until they reached 170 kg regardless of the months of age (HP170, 27 animals). This set up was devised to study separately the effect of weight and age of the animal (characteristics required by the production specifications) on the final product.

The animal ethics committee of the University of Padova (Organismo preposto per il Benessere Animale, OPBA) approved all the experimental procedures performed in this study (approval document #36/2018).

2.2. Ham processing

The slaughtering phase was carried out in accordance with commercial practices. All the obtained thighs were sent to the ham factory ("Attilio Fontana prosciutti", Montagnana (PD), Italy) to be processed into dry-cured hams according to the production specification of "Prosciutto Veneto PDO" (European Commission, 1992). The curing process is described in detail in Toscano et al. (2023). After final trimming, the thighs were completely covered with sea salt (the same for all the hams) and rested at a temperature of 3 °C, for a total duration of 11–14 days depending on the weight of the ham. Once the salt was removed, the hams were first pressed to complete bleeding and salt absorption and then perforated at the distal part of the leg to be hung in a room at 1–3 °C for two weeks. At the end of this phase, called "pre-resting", the hams were moved to a refrigerated chamber at 2–4 °C for 90 days for the "resting" phase.

Subsequently, the hams were washed with water at 40 °C to remove the remaining salt on the surface, prevent crust formation, and trigger proteolysis, and left overnight at 20 °C in a high-humidity room. After one night, the thighs were moved for 40 days into a chamber set at 12–16 °C for the "pre-seasoning" phase. Before proceeding to the 12th-month curing stage, ham parts not covered by the skin were grouted with a dough made of rice flour and lard (all hams received the same dough) to avoid crust formation and to guarantee that the hams were of uniform firmness when sliced.

2.3. Dry-cured ham sampling, DNA extraction, and next-generation sequencing

Once the desired stage of seasoning was reached (about 12 months, during the curing stage), 73 hams were randomly selected. From these, about 5–6 g of product were taken from the surface of each ham at the fat vein, located along the edge of the *semimembranosus* and *quadriceps* muscles, using a sterile blade for each sample. The samples thus obtained were stored at −80 °C until analysis.

At the time of analysis, the samples were thawed, and from each of them a smaller portion of approximately 200 mg was taken through the use of a sterile blade, placed in an amount of RLT buffer (Qiagen, Hilden, Germany), whose volume was set by measuring the exact weight of each slice and adding 2 μL of buffer per mg of tissue, and homogenized using 5 mm stainless steel beads (Qiagen, Hilden, Germany) in a TissueLyser II (Qiagen, Hilden, Germany) for 10 min at 30 Hz. From the homogenized sample, 100 μL were transferred into a sterile 2 mL Eppendorf tube, with 400 μL of RLT buffer (Qiagen, Hilden, Germany). After homogenization, samples were incubated in a thermal block firstly with 20 μl of lysozyme (20 mg/mL; Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 1 h and then with 20 μl of Proteinase K (20 mg/mL; Invitrogen, Carlsbad, CA, USA) at 50 °C for 1 h. Samples were centrifuged at 21000 *g* for 6 min and the supernatant was transferred for subsequent purification steps. DNA was then automatically purified using the Biosprint 96 workstation (Qiagen, Hilden, Germany). This method involves the preparation of 5 96-well s-block plates (Qiagen, Hilden, Germany). Plate number one contains 200 μl of Isopropanol, 20 μl of the MagAttract magnetic beads suspension (Qiagen, Hilden, Germany) plus 300 μl of sample supernatant. Plate number two contains 500 μl of RPW buffer (Qiagen, Hilden, Germany), plates three and four 500 μl of 96% ethanol, and plate five 500 μl of 0.02% Tween solution. DNA was eluted in 200 μl of nuclease free water.

2.4. Library preparation and hypervariable region amplification

The 16S Ion Metagenomics Kit protocol (Thermo Fisher Scientific, Waltham, MA, USA) was used for library preparation. This kit involves the amplification, in two separate PCR reactions, one for the regions V2, V4, V8 and the other for the V3, V6-7, V9, with the following program: 95 °C for 10 min, 25 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 20 s, and a hold stage at 72 °C for 7 min. The amplified hypervariable regions were pooled together and diluted to obtain a concentration of 30 ng μl^{-1}. Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to ligate sample barcodes and amplify the library. This ligation step was done using a QuantStudio 12K-Flex thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA) with two steps at 25 °C for 15 min and 72 °C for 5 min, then library amplification was performed with the following protocol: 95 °C for 5 min, then 7 cycles of 95 °C for 15 s, 58 °C for 15 s, and 70 °C for 1 min. Library quantification was performed with Qubit™ DNA HS Assay Kit on Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The steps of template preparation and sample enrichment were performed respectively with the Ion One Touch 2 and Ion One Touch ES system

(Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Sequencing run on a 520 Ion chip was carried out on Ion Gene Studio S5 (Thermo Fisher Scientific, Waltham, MA, USA) using 850 flows for 400-bases-read sequencing.

2.5. Sequence processing and analysis

Data analyses were performed using the R software (version 4.2.2, https://www.r-project.org/). Preprocessing and filtering of the raw sequences were done with *dada2* (Callahan et al., 2016), using the following criteria: trimLeft = 15 and truncLen = 200. The maxEE of 2 was assigned for the reads. Sequences were dereplicated and chimeras removed using *derepFastq* and *removeBimeraDenovo* functions of *dada2* package, respectively. Only one sample was removed due to the low sequencing depth. Taxonomic assignment of Amplicon Sequence Variants (ASVs) was done using Silva SSU (v. 138.1) (Quast et al., 2013). Specifically, "silva_nr99_v138.1_train_set.fa.gz" was used as the reference dataset, and "silva_species_assignment_v138.1.fa.gz" was used for species assignment. For the nomenclatural version see the note in the legend of Table 1. After taxonomic assignment, unassigned ASVs were removed from the dataset. A threshold of minimum occurrence of a given read = 10 was the conservative cutoff adopted to minimize technical errors of the sequencer. Subsequently, the average and total prevalence of each ASVs (abundance of that sequence variant divided by the total number of sequence variants found in the samples) were calculated to remove the less representative ones. An additional quantitative filter for presence across samples was applied to the resulting dataset (presence threshold = 0.05, i.e. retaining variants that were observed in at least 5 % of the samples).

Table 1
Relative percentage of principal phyla and genera identified in the 72 samples of dry-cured ham.

Phyla and genera	%
Firmicutes	90.82
Tetragenococcus	60.16
Ligilactobacillus	12.63
NA[a]	9.52
Staphylococcus	8.49
Others[b]	0.03
Actinobacteriota	**6.24**
Brevibacterium	3.64
Corynebacterium	1.20
Yaniella	0.67
NA[a]	0.40
Rhodococcus	0.19
Others[b]	0.14
Proteobacteria	**2.71**
NA[a]	0.74
Burkholderia-Caballeronia-Paraburkholderia	0.54
Halomonas	0.44
Herbaspirillum	0.21
Sphingomonas	0.15
Others[b]	0.63
Bacteroidota	**0.12**
Mucilaginibacter	0.11
Others[b]	0.01
Other phyla (17)	0.11

Note: As regards the microbial annotations nomenclature, the output of the latest version of the SILVA Database is reported throughout this manuscript in order to allow uniform bioinformatics comparisons in relation to the stated methods and for the purpose of future meta-analyses. Nonetheless in literature emendments, given taxa names have been substituted (among which: Bacillota for Firmicutes, Actynomycetota for Actinobacteriota, and Pseudomonadota for Proteobacteria).

[a] NA: Genus *N*ot *A*ssessed: within the phylum annotation obtained by the pipeline, the further ranks in the output (not shown) did not reach the genus level.

[b] Others: sum of low percentage bacteria (<0.01 %).

2.6. Diversity indices, core microbiome, and peculiar communities

A dataset reduced to 40 dry-cured hams was used for the evaluation of diversity indices, in order to ensure a balanced representation per rearing strategy (ten animals per group) and sex.

The α-diversity was calculated using the Shannon and Simpson indexes. The α-diversity values were then analysed using the *lme4* (Bates et al., 2015) package within the R environment, using the following linear model:

$$y_{ij} = \mu + rearing\ strategies_i + e_{ij}$$

where y_{ij} was the observed trait (value of Shannon or Simpson index); μ was the overall intercept of the model, rearing strategies$_i$ was the fixed effect of the i^{th} rearing strategy (i = 4; LP, C, HP9M, HP170), and e_{ij} was the random residual. The random residual effects were assumed to be independently and normally distributed with a mean of zero and variance σ^2_e, respectively. The three degrees of freedom due to the rearing strategies were used in orthogonal contrasts to test mean differences of alternative treatments compared to the conventional one (LP vs C, HP vs C) and possible differences within the two levels of HP (HP9M vs HP170). The weighted-UniFrac distance was used to assess β-diversity and the resulting distance matrices were visualized using principal coordinate analysis (PCoA). The result was tested with a PERMANOVA, with the function *adonis2* (*vegan* package, 9999 permutations; Oksanen et al., 2022) and package *pairwiseAdonis* was used to perform the pairwise comparison among rearing strategies (9999 permutations). Furthermore, the homogeneity of dispersion of the four treatments was tested using the *betadisper* function of the *vegan* package, and the results were tested with ANOVA. The core microbiome was evaluated at the phylum and genus level, using a Venn diagram to visualize shared and not shared phyla and genera among the different rearing strategies. Moreover, the statistical associations between phyla/genera and the different rearing strategies were assessed using the function *multipatt* (999 permutations) of the *indicspecies* package (De Cáceres and Legendre, 2009).

The differences were considered significant for a *P*-value <0.05.

3. Results

3.1. Data pre-screening and selection of relevant members of the microbiomes

In the present investigation, 73 dry-cured hams aged 12 months were collected and processed for 16S rRNA sequencing and subsequent analysis. Library sequencing generated a total of 10,471,229 reads, with an average of 143,441 reads per sample. Following dereplication and chimera elimination, the count was reduced to 7,913,065 total reads, with an average of 108,398 reads per sample. The taxonomic assignment of these sequences using reference databases revealed a total of 4468 ASVs, with an average of 62 per sample. Considering the study's objectives, which were to characterize the bacterial population of dry-cured hams subjected to different rearing strategies, and to detect meaningful/significant taxa, the prevalence and abundance-based criteria outlined in the methods section were applied to remove non-representative ASVs. Fig. 1 visually depicts the results for each of the 22 phyla detected. The subsequent choice resulted in a final dataset that included 2845 ASV in 72 samples.

3.2. Overall microbiota profile of dry-cured ham

Due to the prescreening cut-off-based selection, Table 1 displays the phyla and their respective genera that qualified as representative in all the 72 dry-cured ham samples. In practice, one phylum (Bdellovibrionota) was removed after filtering for low abundance, along with 1623 ASVs across different ranks. Firmicutes were the phylum with the

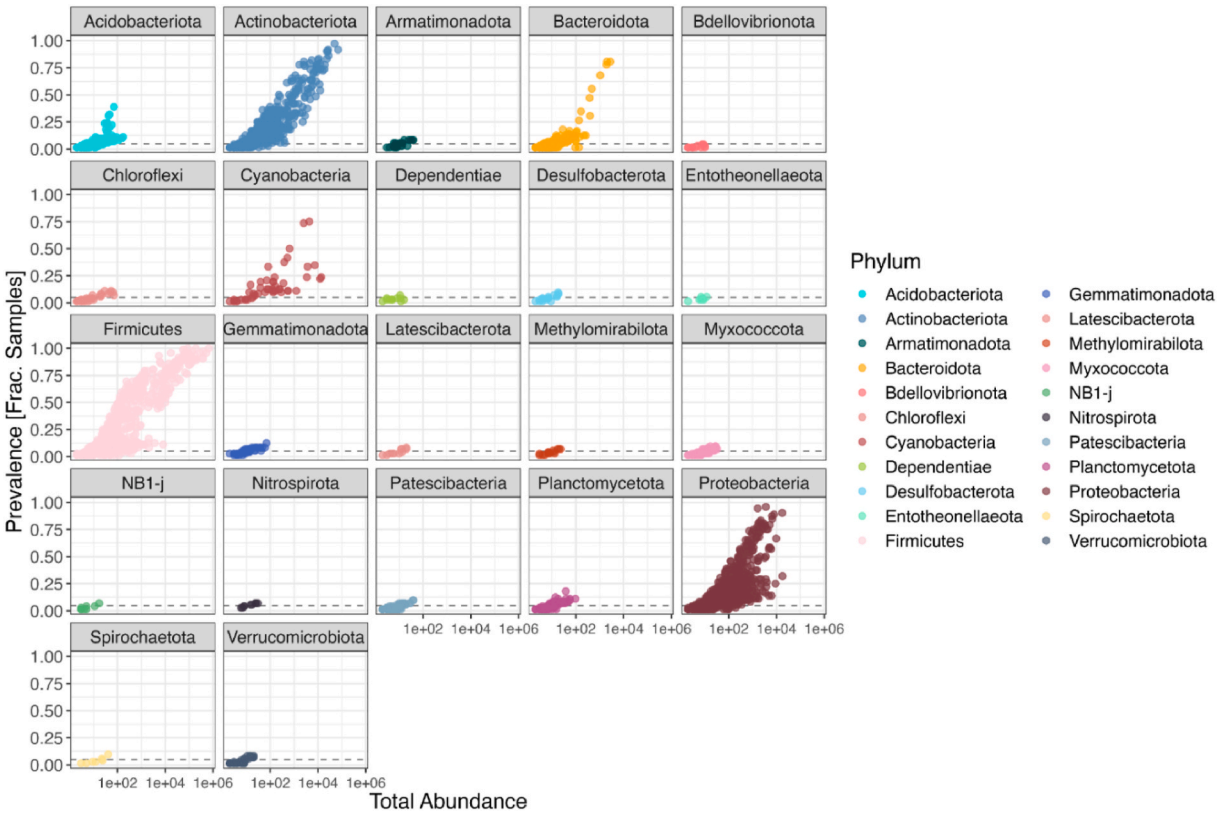

Fig. 1. Total abundance and prevalence of phyla of 72 dry-cured hams after 12 months of seasoning. The horizontal dashed lines mark the (5%) prevalence threshold used for filtering.

highest abundance (90.8%). Actinobacteria (6.2%), Proteobacteria (2.7%), and Bacteroidota (0.12%) were also found in small quantities. *Tetragenococcus* was the most common genus (60.1%), followed by *Ligilactobacillus* (12.6%), *Staphylococcus* (8.5%), *Brevibacterium* (3.6%), and *Corynebacterium* (1.2%).

3.3. Bacterial diversity in dry-cured hams with different rearing strategies

As the varying number of samples within each group of the 72 analysed hams could potentially have an impact on the statistical analysis, we balanced the dataset to ensure equal representation of raising strategies and sex distribution. This process resulted in a small

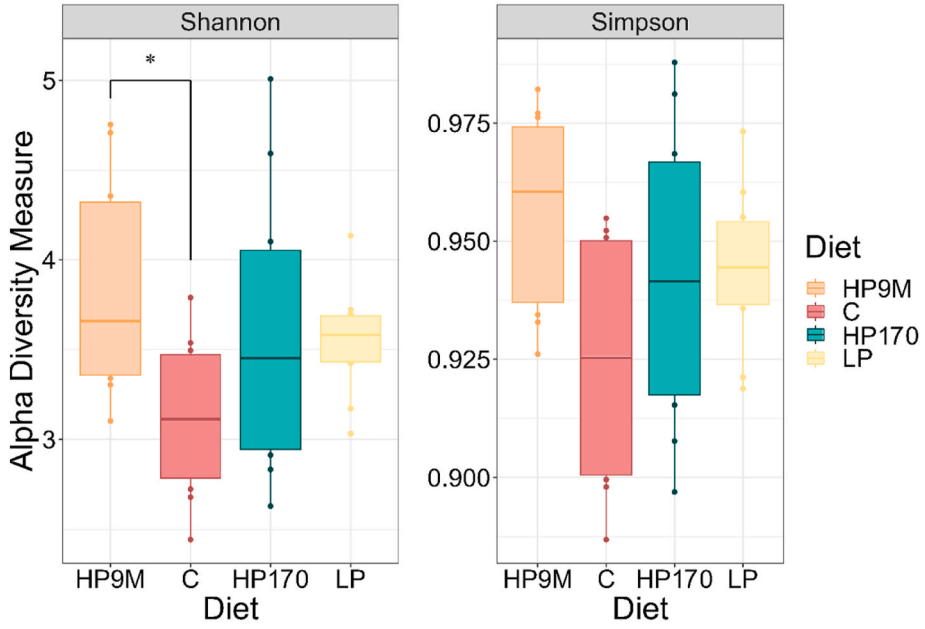

Fig. 2. α-diversity calculated by Shannon (A) or Simpson (B) indexes grouped by diet. Significant contrasts for ANOVA are highlighted, and the *P*-value is indicated by the number of asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). C: control rearing strategy; LP: restrictive low protein diet; HP9M: *ad libitum* high protein diet (9 months of age); HP170: *ad libitum* high protein diet (170 ± 10% kg of weight).

dataset of 40 samples. Shannon and Simpson indices for α-diversity were calculated using this dataset. The analysis of variance (ANOVA) results for α-diversity calculated with the Shannon index revealed that the HP9M group had a higher degree of diversity compared to the C group ($P = 0.0210$; Fig. 2A). There was no significant difference between the HP170 and LP groups. Although the rearing strategies differ significantly ($P = 0.050$), no group differed significantly from the others (Fig. 2B). Fig. 3 shows the diversity across groups, calculated with weighted UniFrac distances, and visualized via PCoA. A PERMANOVA was performed on the calculated distances, and the rearing strategies showed significant differences ($P < 0.001$). The first axis covered 56.7% of the variance, whereas the second axis explained 14.6%. The paired analysis of rearing strategies showed significant differences between the HP170 and C groups ($P = 0.006$), HP170 vs LP groups ($P = 0.048$), and HP9M vs C ($P = 0.006$).

The ANOVA performed on the results of the β-dispersion test showed no significant differences between the treatments ($P = 0.14$), as well as after a permutation test for F-value ($P = 0.125$; 99 permutation).

The phyla and genera were then grouped into four Venn diagrams to visualize their distribution across the four different rearing strategies before (Fig. 4A and B, respectively) and after (Fig. 4C and D) the multilevel pattern analysis, which is a method suited to trace associations between species and groups of sites (De Cáceres and Legendre, 2009). At the phylum level, the HP170 and C groups were associated with only one phylum (Planctomycetota and Acidobacteriota, respectively), one phylum was shared by HP9M and LP (Bacteroidota), one phylum was shared by HP9M, HP170, and LP (Actinobacteriota), and two were shared by all rearing strategies (Firmicutes and Proteobacteria). The LP group had six distinct genera (*Candidatus* Cardinium, *Corynebacterium, Garicola, Halomonas, Kocuria, Sphingomonas*), four for HP170 (*Gemmata, Salinicoccus, Serratia, Yaniella*), three for C (*Edaphobacter, Occallatibacter, Ochrobactrum*), and twelve for HP9M (*Arthrobacter, Brochothrix, Caulobacter, Izhakiella, Kosakonia, Ligilactobacillus, Micrococcus, Photobacterium, Pseudoalteromonas, Psychrobacter, Salinibacter* and *Stenotrophomonas*). Among the peculiar genera, some were found to be common in different treatments. The HP9M and LP shared the *Staphylococcus* genus; HP9M - LP - C shared the *Tetragenococcus* genus; and HP170 – HP9M - LP shared the *Brevibacterium*. In Table 2, the statistically associated genera for each treatment, along with their relative percentages, are presented.

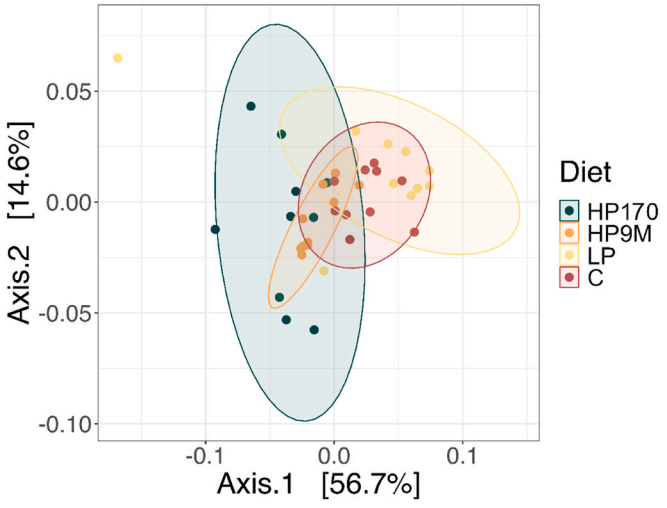

Fig. 3. β-diversity calculated using weighted UniFrac distance. Significant differences were found between the HP170 and C groups (P = 0.006), HP170 vs LP groups (P = 0.048), and HP9M vs C (P = 0.006). C: control rearing strategy; LP: restrictive low protein diet; HP9M: *ad libitum* high protein diet (9 months of age); HP170: *ad libitum* high protein diet (170 ± 10% kg of weight).

4. Discussion

4.1. Microbiota characterization of dry-cured hams

Dry-cured hams are considered stable matrices due to their production processes, such as salting and low humidity conditions, which impose constraints on microbial growth (Toldrà and Aristoy, 2010). Nevertheless, during the aging period, complex enzymatic processes occur, partly attributed to microbial enzymes contributing to the final aromatic profile of the hams. The microorganisms producing these enzymes have adapted to high salinity and low moisture conditions (Toldrà and Aristoy, 2010).

Several studies assessing the associations of the microbiome with different aspects of Spanish (Cordero and Zumalacárregui, 2000; Martín et al., 2008; Martínez-Onandi et al., 2019) or Chinese (Hu et al., 2009; Li et al., 2022; Zhang et al., 2023) ham production can be found in the literature. In Italy, although processed meat is a prominent production, few works have evaluated these aspects (Busconi et al., 2014; Hinrichsen and Pedersen, 1995). In this study, the microbiota of hams was investigated after 12 months of maturation, as this period is when most of the alterations are expected to occur, particularly around the intersections of muscle and fat. Indeed, water loss during curing can cause the separation of the lean part from the fat, forming small fissures that can serve as an ideal site for undesirable bacteria proliferation (Girón et al., 2015; Untermann and Müller, 1992).

We performed 16S rRNA sequencing of targeted hypervariable (V) regions, which has become the predominant tool for characterizing the composition of bacterial communities (Zhang et al., 2018). However, recent approaches have enabled full-length 16S sequencing, allowing for a more comprehensive characterization of microbial communities, but are often associated with higher error rates with long-read sequencing, and require more resources for data analysis and interpretation, especially given the limited available comparative studies (Callahan et al., 2019). To cope with these issues, particularly concerning the nearly identical V-region sequences among numerous bacterial species (Yang et al., 2016), we sequenced a high number of hypervariable regions (V2, V3, V4, V6-7, V8 and V9) to limit under- or overrepresentation of taxa (Wensel et al., 2022).

Our findings confirmed the presence of populations identified in previous investigations. From Table 1, the majority of microorganisms identified belong to the genus *Tetragenococcus*, a lactic acid bacterium (LAB) capable of surviving in high-salt foods like kimchi, fish sauce, anchovy, pickles, and soy sauce other than dry-cured hams (Martínez-Onandi et al., 2019). *Tetragenococcus* species related to dry-cured ham production are *T. halophilus* and *T. koreensis*: in particular, *T. halophilus* was identified in Italian PDO Coppa and Pancetta Piacentina, which are other types of cured meats derived from pig raw meat ripened for 6 and 3 months, respectively (Busconi et al., 2014). Although the authors did not perform detailed analyses of the species, the 16S taxonomic assignment showed that some ASVs belonged to *T. halophilus* and *T. koreensis* species, as shown in the literature.

Among salt-tolerant bacteria, the genera *Brevibacterium* and *Staphylococcus* have been previously associated with the formation of the hams' aromatic profile (Martínez-Onandi et al., 2019; Virgili et al., 2007). Again, our taxonomic assignment has shown the presence of *S. equorum*, which has already been reported as one of the most associated with the production of dry-cured hams, together with *S. xylosus* (Martínez-Onandi et al., 2019; Rodríguez et al., 1994). In a small proportion, we also detected the genus *Corynebacterium*, which is linked to fermentative processes and has been previously identified on the meat surface during the initial phases of ham processing (Hinrichsen and Pedersen, 1995).

Some of the genera found in this study have been previously associated with the development of defects during the aging process. *Ligilactobacillus*, the second most present genus in our dataset, is a LAB already found in Iberian dry-cured hams (Martín et al., 2008; Zhou et al.,

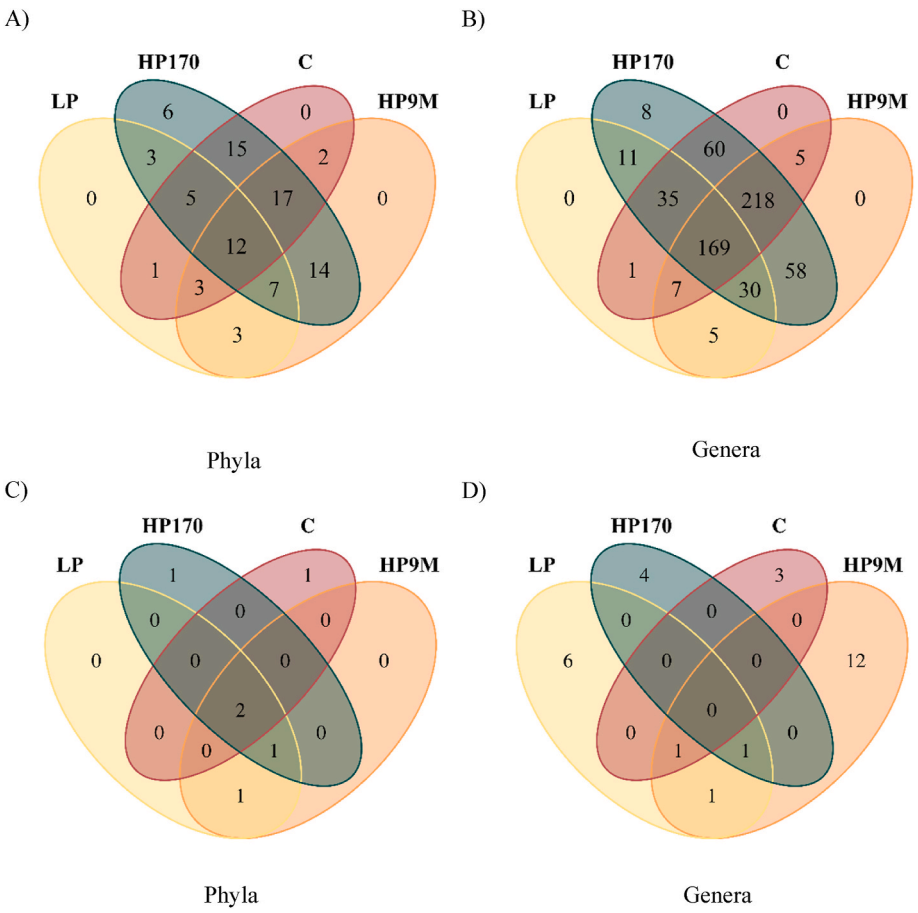

Fig. 4. Venn diagrams showing the core and specific numbers of bacterial phyla (A, C) or genera (B, D) observed on the 40 dry-cured hams subset after 12 months of seasoning. The top panels (A, B) are constructed on the plain dataset values, while the two bottom ones (C, D) are the ones showing the numbers of remaining taxa after running a multilevel pattern analysis (De Cáceres and Legendre, 2009). C: control rearing strategy; LP: restrictive low protein diet; HP9M: *ad libitum* high protein diet (9 months of age); HP170: *ad libitum* high protein diet (170 ± 10% kg of weight).

2022). Specifically, *L. salivarius* was associated with the development of "bone-taint" spoilage, which can occur in the muscle close to the bone and give a foul-smelling odor and pasty texture to meat. Another genus that includes spoilage species is *Burkholderia*. For instance, *B. cepacea* was identified in a study on Iberian hams and classified as a spoilage-causing agent (Martín et al., 2008). Although no visible alterations were detected in the hams in our investigation, as stated previously, the presence of bacterial species with altering capabilities at this stage of maturation is not surprising. In fact, as curing progresses, the moisture content of the hams drops with a gradual increase in NaCl concentration, limiting bacterial growth (Martín et al., 2008). Despite this, further research is needed to elucidate whether and how species belonging to the genera *Ligilactobacillus* and *Burkholderia* may exert their roles as spoilage agents in dry-cured hams at the end of the curing process.

Furthermore, it might be noteworthy to mention that, given that the data used in our investigation came from a single ham factory, this factor may have also had some influence on the microbial profile. Nonetheless, studies have shown that different production environments lead to differences in the microbiota of dry-cured hams (Ge et al., 2017).

4.2. Biodiversity of dry-cured hams

The biodiversity among the samples of dry-cured hams was calculated using the Shannon and Simpson indices as measures of α-diversity. A significant difference was found in the Shannon index between the HP9M group (*ad libitum* with a high-protein diet) and the C group (restrictive feeding with a medium-protein diet). The Shannon index takes into account both the species abundance in the sample and their evenness (Jost, 2006), and generally, its values range from 1.5 to 3.5 (O'Keeffe, 2004). In this study, the average values of this index are at or above the value of 3.5 (as in the case of HP170 and HP9M groups) indicating how wide the biodiversity is in our samples even though, as stated earlier, *Tetragenococcus* represents the majority of the population. Concordant to this, Simpson's index values are also very high in all the rearing strategies groups. A higher value of Simpson's index indicates greater diversity within the sample (Gregorius and Gillet, 2008). Differently from Shannon's index, no significant differences were found among the rearing strategies for Simpson's index.

As depicted in Fig. 2A and B, most of the biodiversity is shared between two or more rearing strategies. We can assume that, despite the differences found in the chemical and morphological characteristics of hams at the end of curing (Toscano et al., 2023), the seasoning process and salt content reduce the variability of α-diversity across the four rearing strategies.

The results of the PERMANOVA test on distances calculated by the weighted UniFrac method showed that the effect of rearing strategies is significant. This means that the centroids of the different groups are far enough apart to distinguish the effect of the rearing strategies (Fig. 3). It is worth mentioning that the method used to calculate distances takes into account not only the composition and abundance of species but also on the phylogenetic relationships of individuals (Chang et al., 2011). The groups HP170 and HP9M, both fed *ad libitum* with a higher protein content than C (16.2% vs. 12.8% crude protein content, respectively), are almost totally overlapping (with the HP170 group having a greater dispersion) while the C group is positioned intermediately between the

Table 2
Relative percentage of peculiar communities statistically associated with individual rearing strategies in the 40 dry-cured hams samples subset.

Phylum and Genus	Rearing strategies				Group	Potential Effect	Reference
	C[a]	LP[b]	HP9M[c]	HP170[d]			
Firmicutes, %							
Tetragenococcus	12.1487	0.0013	9.763	–	Favourable	aromatic profile formation	Martínez-Onandi et al. (2019)
Ligilactobacillus	–	–	10.8837	–	Spoilage	"bone taint" off odors	Zhou et al. (2022)
Staphylococcus	–	0.0084	0.0187		Favourable	aromatic profile formation	Martínez-Onandi et al. (2019)
Salinicoccus	–	–	–	0.0006	Favourable	correlated with various metabolites	Zhu et al. (2021)
Brochothrix	–	–	0.0041	–	Spoilage	off odors and off color formation, found in RTE (ready to eat) ham slice	Reichel et al. (2020)
Actinobacteriota, %							
Brevibacterium	–	0.1725	0.1608	0.0259	Favourable	aromatic profile formation	Virgili et al. (2007); Busconi et al. (2014)
Corynebacterium	–	0.1348	–	–	Favourable	color and odor formation	Hinrichsen and Pedersen (1995); Li et al. (2022)
Yaniella	–	–	–	0.0854	Unknown	no association with food in the literature	
Kocuria	–	0.0004	–	–	Favourable	isolated in salt	Cordero and Zumalacárregui (2000)
Garicola	–	0.0008	–	–	Unknown	no association with food in the literature	
Micrococcus	–	–	0.0203	–	Favourable	isolated in salt	Cordero and Zumalacárregui (2000)
Arthrobacter	–	–	0.0028	–	Spoilage	environmental contaminant	Hu et al. (2009)
Proteobacteria, %							
Halomonas	–	0.0442	–	–	Favourable	aromatic profile formation	Zhu et al. (2021); Zhang et al. (2023)
Sphingomonas	–	0.0002	–	–	Favourable	production of acids and alcohols	Zhang et al. (2023)
Ochrobactrum	0.0038	–	–	–	Favourable	production of acids and alcohols	Zhang et al. (2023)
Caulobacter	–	–	0.0002	–	Unknown	founded in a nutrient-rich water environment	Wilhelm (2018)
Psychrobacter	–	–	0.0164	–	Unknown	psychrophilic, not associated with specific function during seasoning	Zhang et al. (2023)
Izhakiella	–	–	0.0023	–	Unknown	no association with food in the literature	
Kosakonia	–	–	0.0003	–	Unknown	no association with food in the literature	
Stenotrophomonas	–	–	0.0001	–	Unknown	psychrophilic, not associated with specific function during seasoning	
Serratia	–	–	–	0.0005	Spoilage	off odors and off color formation	Belletti et al. (2013)
Pseudoalteromonas	–	–	0.0014	–	Spoilage	environmental contaminant	Zhu et al. (2021)
Photobacterium	–	–	0.0009	–	Spoilage	environmental contaminant	Zhu et al. (2021)
Acidobacteriota, %							
Edaphobacter	0.0018	–	–	–	Unknown	no association with food in the literature	
Occallatibacter	0.0005	–	–	–	Unknown	found in soil	Foesel et al. (2016)
Bacteroidota, %							
Candidatus Cardinium	–	0.0022	–	–	Spoilage	found in parasites of food products	Hubert et al. (2012)
Planctomycetota, %							
Gemmata	–	–	–	0.0002	Unknown	no association with food in the literature	

[a] C: control rearing strategy.
[b] LP: restrictive low protein diet.
[c] HP9M: *ad libitum* high protein diet (9 months of age).
[d] HP170: *ad libitum* high protein diet (170 ± 10% kg of weight).

high-protein and the low-protein strategies (LP, 11.3% crude protein content). The pairwise comparison highlighted that, in terms of microbial composition, HP170 differs from C and LP and HP9M differs from C. As reported in Toscano et al. (2023), the different protein content of the four strategies and the different feeding regimens (restrictive vs *ad libitum*) firstly influenced the morphometric characteristics of the fresh legs and consequently the chemical characteristics of the dry-cured ham. We can hypothesize that differences in terms of the percentage content of protein and/or lipids, variations in moisture content, and even the different weight of fresh hams (which reflects the available surface area) might have created different environments for microbial growth.

In Fig. 4C and D, and Table 2, the statistical association of phyla and genera with different rearing strategies are reported. This statistical approach (De Cáceres and Legendre, 2009) allowed to exploit the relative abundance and relative frequency of the taxa occurrence to investigate the association among the most characteristics taxa and the different rearing strategies. At the genus level, we can observe the presence of genera that have been already associated with various stages of ham seasoning and that are active in the formation of the traditional aromatic profile of dry-cured ham. Indeed, we observed the presence of *Tetragenococcus* (associated with C, HP9M, and LP), *Brevibacterium* (associated with LP, HP9M, and HP170), and *Staphylococcus* (associated with HP9M and LP). As previously described, all of these are salt-tolerant bacteria found in meat-based processed products that contribute to the aromatic profile formation (Martínez-Onandi et al., 2019; Virgili et al., 2007).

When considering each specific rearing strategy, the LP group was found to have the highest number of favourable bacteria. As observed by Toscano et al. (2023), this rearing strategy was found to be the least favourable for dry-cured ham production from a technological point of view. The reason for this association might be the higher lipid and the lower protein contents in the lean part of the hams. Studies showed that a reduction in dietary protein content leads to greater activation of lipogenic enzymes in muscle than in subcutaneous fat, hence increasing the lipid content in the lean part of hams (Schiavon et al., 2015; Wood et al., 2013). This may have provided a favourable substrate for the development of bacteria such as *Corynebacterium*, *Sphingomonas* , and *Halomonas* (Li et al., 2022; Zhang et al., 2023; Zhu et al., 2021). It is worth noting that the LP group is also associated with *Candidatus* Cardinium, a spoilage bacterium: its presence is frequently associated with synanthropic mites, which are a source of contamination for dry-cured hams (Hubert et al., 2012).

The HP9M group was worthy of note. This group had the most relationships with different genera and was the one that exhibited the best technological qualities in the study of Toscano et al. (2023). The larger ham size and highest dietary protein content may have offered a

favourable environment for the growth of the numerous peculiar communities associated with this rearing strategy. However, many of the associated genera belonged to the spoiling group. This group includes several bacteria that are considered environmental contaminants, such as *Pseudoalteromonas* and *Photobacterium*, which are generally associated with marine environments but are recognized in the literature as spoilage agents in hams (Zhu et al., 2021). Among those defined as environmental contaminants, *Arthrobacter* was also present and was associated with the slaughter phase (Hu et al., 2009). *Ligilactobacillus* and *Brochothrix* have been identified directly in dry-cured hams; the former is among the most present in these rearing strategies (10.9%) and leads to the formation of "bone-taint" off odors (Zhou et al., 2022), while the latter has been observed in ready-to-eat sliced hams and, in high concentrations, can even cause gastrointestinal problems (Reichel et al., 2020). The HP9M group also had the largest number of unknown bacteria. Some, such as *Psychrobacter* and *Stenotrophomonas* , are psychrophilic bacteria and are not associated with specific functions in hams (Zhang et al., 2023), others, such as *Izhakiella* and *Kosakonia* have not been found associated with any food matrix. Finally, *Caulobacter* was found in nutrient-rich water (Wilhelm, 2018) and could be derived from water used at slaughterhouse or ham factory.

Given the number of unknown or spoilage genera in our research and given recent revisions to production specifications that would allow consortia to increase slaughter weights as simulated in the HP9M group, it is important to further investigate this rearing strategy. This should include conducting studies at the end of the ham maturation period to evaluate whether the presence of spoilage genera is maintained or if the complete maturation of ham reduces or eliminates the issue.

Finally, the C and HP170 groups, whose ham characteristics were found to be similar (Toscano et al., 2023), both reported the lowest number of associations with unique communities, evenly divided between favourable and unknown. Only for the HP170 strategies, an association with *Serratia*, a genus belonging to the spoilage group, was observed.

It is important to highlight that the sum of favourable, spoilage, and unknown genera account for 33.5% of all the genera found in our samples. This percentage is primarily represented by *Tetragenoccus* (22%) and *Ligilactobacillus* (10.8%), with the former belonging to the favourable group and the latter to the spoilage group. All other genera are present in a small percentage; therefore, more investigations are needed to assess the impact of these genera on the chemical, physical, and sensory characteristics of hams at the end of the seasoning.

5. Conclusion

This study characterized the microbiota of Italian PDO dry-cured hams produced using three alternative rearing strategies compared to the control one. The results highlighted that the HP9M rearing strategy differed in terms of α-diversity from C. As for β-diversity, the PERMANOVA highlighted significant differences in the microbial communities. However, they have shown a common core, consisting of three genera *Tetragenococcus*, *Brevibacterium*, and *Staphylococcus*, representing 72.2% of the relative abundance of all genera and contributing to the formation of the aromatic profile of dry-cured hams, concordant with previous findings. The rearing strategy with the largest number of peculiar communities was HP9M, which is associated with the production of the best dry-cured ham. Despite this, the HP9M strategy showed most of the spoilage and unknown bacteria, for which further controls are therefore needed. Future studies should investigate whether observed differences in the microbiota of different rearing strategies are able to modulate the chemical and physical characteristics of dry-cured hams during the production process and possible associations with flavour compounds typical of the sensory profile of dry-cured hams.

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CRediT authorship contribution statement

Alessandro Toscano: Writing – original draft, Visualization, Investigation, Formal analysis. **Diana Giannuzzi:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Isaac Hyeladi Malgwi:** Writing – review & editing, Writing – original draft, Data curation. **Saptharati Deb:** Writing – review & editing, Software, Formal analysis, Data curation. **Chiara Broccanello:** Writing – review & editing, Data curation. **Andrea Squartini:** Writing – review & editing, Validation, Supervision, Methodology, Investigation. **Piergiorgio Stevanato:** Writing – review & editing, Software, Resources. **Alessio Cecchinato:** Writing – review & editing, Resources, Funding acquisition. **Luigi Gallo:** Writing – review & editing, Resources, Funding acquisition. **Stefano Schiavon:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors have no conflict of interest to declare.

Data availability

The data that support the findings of this study are available in the NCBI Sequence Read Archive (SRA) repository under the accession number [PRJNA1077237].

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