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Impact of dry aging on quality parameters and microbiological safety of beef

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ABSTRACT

The market of dry aged meat is rapidly increasing. However, few papers report the impact of specific dry aging parameters on quality and microbiological safety of dry aged meat. In this study we assessed the impact of a 90 days dry aging process, performed in a commercial cabinet, on the quality parameters and microbiological safety of Holstein beef loins. Meat pH, colour indexes and fatty acids were stable during the process. Main volatile compounds' concentration increased over time. Any oxidation of protein residues was detected and the main proteins undergoing proteolytic degradation early in the process were myoglobin, myofibrillar constituents and sarcoplasmic enzymes. The most significant histological changes started at 30 days and striation impairment was observed, not related to oxidative processes. The total bacteria count increased without signs of spoilage, Enterobacteriaceae were not enumerated and few reads of foodborne pathogens were detected by shotgun metagenomic. These results show that when the dry aging process is performed in a dedicated cabinet under strictly controlled process parameters, the microbiological safety of the meat is preserved. Our study provides the first insights of dry aged Holstein loins, whose valorisation would certainly enhance the economic, social and environmental sustainability of the Parmigiano Reggiano food system.

1. Introduction

The per capita consumption of beef meat in Europe is expected to drop from 69.8 kg in 2018 to 67 kg by 2031 (DG-AGRI European Commission. Directorate General for Agriculture and Rural Development, 2021). Based on EU Reg 853/2004 "fresh meat" is defined as "meat that has not undergone any preserving process other than chilling, freezing or quick-freezing, including meat that is vacuum-wrapped or wrapped in a controlled atmosphere". Regulation 852/2004 includes in the 'processing' definition any action that substantially alters the initial product, including heating, smoking, curing, maturing, drying, marinating, extraction, extrusion or a combination of those processes. Technologies for meat maturation, such as dry-aging, are gaining popularity (Dashdorj et al., 2016) because meat consumers are asking for premium quality beef products with characteristic sensory attributes (Álvarez et al., 2021). The dry aging process determines development of characterized and intense flavour based on the activity of endogenous proteolytic enzymes (as for example calpains or aminopeptidases) that cleave peptidic bonds in muscle proteins, leading to a degradation of the muscle structure and increasing the tenderness of the meat as the aging process advances. In addition, not only meat becomes more tender but also exhibits more pronounced organoleptic qualities.

There is a growing interest with respect to dry aged meat in the United States, Australia, Asian and European Countries (Dashdorj et al., 2016; Hulánková et al., 2018; Ryu et al., 2020). This interest led enterprises, such as slaughterhouses, meat shops, and restaurants, to produce and sell dry-aged beef (Rezende-de-Souza et al., 2021) mostly using commercial availability of small maturation chambers. The use of such maturation facilities, also among non-professional food business operators, poses a raising problem of microbiological safety. The

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microbiological safety of dry-aged meat has recently been addressed in an EFSA scientific opinion (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2023). Dry aging is described as "the process carried out in aerobic conditions of hanging beef carcasses or subprimal or placing primal cuts either unpacked or packed in bags permeable to water vapour in a refrigerated room and left to age for several weeks or even months at controlled environmental conditions of temperature, relative humidity and air flow". However, there is a lack of standardization in the setting of process parameters used for the meat dry aging and a poor control of such parameters, mostly represented by temperature, ventilation and relative humidity, within the maturation chamber (Gowda et al., 2022; Lancaster et al., 2022; Rezende-de-Souza et al., 2021).

Several factors affect meat quality parameters and microbiological safety as well as process yield of dry-aged beef, as type of raw material (*i.e.*, animal age, sex, weight, breed) amount of subcutaneous and intramuscular fat and type of cuts. The cuts that are primarily used for meat dry aging are obtained from the thoracic lumbar region bone-in and subjected to ageing from 3 to about 8 weeks, but up to 290 days of ageing are reported (Smaldone et al., 2019). The homogeneous fat cover, cut size and shape, and the presence of bones may explain the preference for this cut for the dry aging process as it reduces weight losses due to ageing and the amount of meat discharged during the trimming of the crust. Restaurants and butcher shops usually receive raw material either wrapped in different packing materials or unwrapped, but also vacuum packed.

From a microbiological point of view the characteristics of meat prior to aging, hygienic practices during production, time and preservation of the meat between slaughter and the start of the dry aging process, process parameters and storage conditions will affect the safety of dry-aged beef (Gowda et al., 2022; Khazzar et al., 2023; EFSA Panel on Biological Hazards (BIOHAZ) et al., 2023). Indeed, both quality and safety of dry aged meat are conditioned by the interaction of different variables, whose specific effect is scarcely investigated.

Therefore, the objective of this study was to investigate the quality parameters and microbiological safety of dry aged meat marketed in Italy. To have a preliminary overview of the microbiome associated to dry aged meat, at each sampling point one surface sample was investigated by shotgun metagenomic. Considering the raise of habit to perform the dry ageing of meat in restaurants and shops, we have focused our analysis on the effect of dry aging on loins of Holstein cattles from the Parmigiano Reggiano supply chain. The latter has been chosen considering its possible valorisation, since culling in the dairy sector provides a consistent number of animals per year for meat production (Piazza et al., 2023).

2. Materials and methods

2.1. Experimental plan

A total of 3 loins obtained from three different Holstein cattle from the Parmigiano Reggiano supply chain were purchased and tested in this study (Fig. 1). Each loin, of approximately 5 kg, was considered a sample unit. The Holstein loins came from cow carcasses of approximately 360 kg and a mean age of 45 months. After slaughtering, the half carcass of the tested Holstein was cold stored (0–3 °C) for 4 days. Then the three loins for the trial were removed from each carcass and put the same day in the maturation room at the processing facility. The tested loins were of normal bloomed beef colour and without quality defects. The maturation room was a Stagionello® Meat Curing Device (Crotone, Italy), an equipment designed and patented for meat dry aging following pre-set parameters: temperature of 1 °C, airflow speed of 1.8 m/s, and relative humidity of 78%.

Samplings of each meat loin were performed at day 0, 30, 45, 60 and 90 (identified as T0, T1, T2, T3 and T4) of maturation. Two types of samples were collected at each sampling point: surface samples and inner samples. The surface samples consisted of 10 g of the exposed

Fig. 1. Image of the three loins tested in this study.

muscular part of the loins measuring 3±2 mm thickness. Inner samples were collected from the deep part of the loins with at least 10 cm thickness cutted off with a bone saw in order to remove all the crust (about 1–2 cm). The final sample was about 3 cm thick. All samples were preserved at 0–4 °C during transport from the processing facility to the laboratories. The surface sample of each loin was tested for total bacteria counts (TBC) and Enterobacteriaceae (EB) at all sampling points (n = 15). Moreover, the inner sample of one loin was also tested by shotgun metagenomic at each sampling points (n = 5). The inner sample of each loin was tested for the quality parameters and consumers' sensory evaluation at T0, T1, T2, T3 (n = 12). Moreover, the inner sample of one loin was submitted to histological evaluations at all sampling point (n = 5) obtaining at least 10 histological sections for sample as described below.

2.2. Analyses of the quality parameters

One inner sample for each loin was obtained after dried surfaces (crust) removal by trimming, in order to simulate the real scenario, and tested for chemical, sensorial and biochemical parameters during aging time T0, T1, T2 and T3. All chemical reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany). The internal standard for volatile compounds profile (VOCs) analyses (4-methyl-2-pentanone; CAS 108-10-1) was also purchased from Merck (Darmstadt, Germany). For fatty acid analysis, a 37 component FAME mixture in dichloromethane was obtained from Supelco (Supelco, Bellafonte, PA, USA). Stock solutions of fatty acid standard compounds were prepared in hexane at a concentration of 10 mg mL−1, and stored at −20 °C. Further standard dilutions were individually prepared in hexane or in mixtures to achieve a final concentration of between 0.2 and 1.0 mg mL−1 prior to gas chromatography (GC) analysis.

2.2.1. pH measurement

The pH measurements were conducted using a pH meter equipped with an insertion electrode (Delta-OHM HD 2105.2), in triplicate in each inner sample collected from each loin, by inserting the electrode directly into the meat.

2.2.2. Colorimetric analysis

The colour assessment was performed by using a Minolta Croma-Meter CR-400 (Minolta Camera Co., Ltd., Osaka, Japan). The measurements were based on the CIE L*a*b* colour coordinates: L* or brightness, with values from 0 to 100 (from black to white), a* or red index, with values from −120 to +120 (with negative values toward green and positive values toward red) and b* or yellow index, with values from

−120 to +120 (with negative numbers toward blue and positive numbers toward yellow). The measurements were taken in triplicate in each loin to consider the inhomogeneity of the sample.

2.2.3. HS-SPME analysis of volatile compounds profile (VOCs)

For the analysis 5g of homogenized sample, representative of each inner sample, were placed into 20 mL headspace vials together with 100 μL of 20 μgmL^{-1} 4-methyl-2-pentanone solution ad internal standard and closed with polytetrafluoroethylene (PTFE)-coated silicone rubber septa (20 mm diameter) (Supelco, Bellefonte, PA, USA). The equilibrium state of the volatile compounds was achieved by leaving the samples at 5 ± 1 °C for at least 1 h before extraction conducted with a conditioned (at 280 °C for 1.5 h) 85 μm Carboxen/polydimethylsiloxane (CAR/PDMS) StableFlex fibre (Supelco, Bellefonte, PA, USA) at 5 ± 1 °C for 120min. For the instrumental analysis an Ultra gas chromatograph TraceGC (Thermo Scientific, San Jose, CA, USA) equipped with an Rtx-WAX column (30 m × 0.25 mm i. d., 0.25 μm film thickness; Superchrom, Milan, Italy) coupled to a TraceDSQII mass spectrometer (Thermo Scientific, San Jose, CA, USA) was used as previously described (Panseri et al., 2011). The mass spectra of the standards, when available, or the ones reported in the National Institute of Standards and Technology (NIST) US Government Library or in Wiley spectral databases were used to confirm the identity of the compounds. The results of the analyses, conducted in triplicate, were expressed as ng g^{-1} internal standard equivalents.

2.2.4. Fatty acid determination

Briefly, 3 g of matrix were extracted according to the (Bligh & Dyer, 1959), subsequently an aliquot corresponding to 20 mg of fat was derivatized according to Christie and Han (2012). The methyl esters of the fatty acid were then analysed by gas chromatography using a gas chromatograph (TRACE GC Ultra, Thermo Fisher Scientific, Rodano, Italy) equipped with a flame ionisation detector (FID). The content of fatty acid methyl esters (FAMEs) was quantified by weight expressed as g/100g of the total FAMEs. A RT-2560 fused silica capillary column (100 m × 0.25 mm x 0.25 μm film thickness; Restek, Milan, Italy) was used with a temperature programmed from 70 °C to 250 °C at 2 °C min-1. The carrier gas was nitrogen at 1.0 mL min-1 with an inlet pressure of 16.9 psi. The content of fatty acid methyl esters (FAMEs) was quantified by weight expressed as g/100g of the total FAMEs. All analyses were done in duplicate.

2.2.5. Peptidomic and proteomic analyses

For peptidomic investigations, approximatively three aliquots of 0.1 g of longissimus dorsi muscle tissues, sampled from three different regions within each loin, were pooled and homogenized using a Varispeed A581 v.220 Potter homogenizer (Orlando Valentini, Milan, Italy) in 1 mL 0.2% formic acid, 0.2% CH3CN at full speed for 1 min, followed by 1 min on ice. The homogenate was centrifuged at 21,428 g for 15 min at 18 °C to pellet the tissue debris. Peptides were enriched and analysed according to Aletti et al. (2016) by a mass spectrometry-based protocol using a Dionex Ultimate 3000 nano-LC system coupled to a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Scientific). Regarding proteomics, the same homogenization protocol described for peptidomics was used except for the homogenization buffer (2 mL 8 M urea, 20 mM Hepes pH 8.0, with Protease inhibitor cocktail). Following dilution of urea to 2M with NH4HCO3, proteins were reduced, alkylated, digested, desalted and analysed according to Maffioli et al., 2020. Nano-LC-MS/MS analysis was performed as described above. Peptides and proteins identification and quantification was performed using Max Quant and Perseus software packaging according to Maffioli et al. (2020). Only peptides present in at least 2 out of 3 biological replicates were considered as positively identified and quantified. Two bioactive peptides databank were used (*i.e.,* BIOPEP https://biochemia.uwm.edu.pl/biopep-uwm/and SATdb http://crdd.osdd.net/raghava/satpdb/).

2.3. Histological evaluations

At each sampling time a specimen of the inner sample was collected in one loin. All samples were fixed in buffered formalin (pH 7.2) for 72 h. After fixation, the samples were washed to remove excess fixative and trimmed in such a way as to obtain, for each sample, portions of muscle with fibers oriented in both transversal and longitudinal directions. After dehydration in an alcohol battery and subsequent clearing in xylene, the samples were embedded in paraffin. At least 10 histological sections (7 μm thick) were obtained from each paraffin block, mounted on poly-L-lysinated slides and subsequently stained with Masson's trichrome.

2.4. Consumers' sensory evaluation

The sensory evaluations were conducted according to (Corbin et al., 2015; Nobile et al., 2024) where a panel of untrained testers was provided with a hedonistic sheet showing the main characteristics of the meat products, which was given a rating between 0 and 9 for the 8 descriptors (colour of lean part-red to brown; colour of fat part-white to yellow; colour uniformity-halos, spots, greening etc.; olfactory intensity; possible off-flavours; sour smell; rancid smell; overall liking). The sensory evaluations have been considered in this study since were focalized to describe possible negative traits (off-flavor) occurred during aging period. Meat slices obtained from each loin at each sampling time were presented to a panel of 20 semi-trained naïve consumers (from 20 to 60 years old, in equal numbers for both genders). Consumers were recruited among Ph.D. students, researchers and professors based on the following: a liking for meat, consuming meat at least once a week (consumer's habit) and having an interest for the study. They were educated with previous training during two sessions evaluating the sensory attributes used for meat evaluations.

2.5. Microbiological analyses

2.5.1. Enumeration of total bacteria counts and enterobacteriaceae by culture methods

At each sampling time, 10 g of surface sample were aseptically collected from different parts of each loin and diluted in 90 mL of sterilized Buffered Peptone Water (PW, CM0009, OXOID, Basingstoke, UK), placed in a sterile stomacher bag and homogenized for 5 min using a stomacher (BagMixer®, Interscience, St Nom, Francia). Subsequently, ten-fold serial dilutions of each homogenate were prepared in PW, followed by spread plating in duplicate for Total Bacterial Counts (TBC) performed according to ISO 4833–2:2013 on Plate Count Agar (PCA; CM0325, Oxoid) incubated at 30 °C for 48/72-h and for the count of the Enterobacteriaceae (EB) performed according to ISO 21528–2:2017 on Violet Red Bile Glucose Agar (VRBG, CM1082, Oxoid) incubated at 37 °C for 24-h.

2.5.2. Shotgun metagenomic analysis of the surface samples

At each sampling time, 10 g of surface meat was aseptically collected from the crust of different parts of one loin with a sterile scalpel. Each sample was then diluted in 90 mL of sterile physiological solution (NaCl 0.9%), homogenized in the stomacher (BagMixer®, Interscience, St Nom, Francia) for 1 min and centrifuged at 4 °C for 20 min at 9980×*g*. The obtained pellet, containing the concentrated cells, was stored at −80 °C until DNA extraction. The DNA was extracted from the pellet using a bead-beating procedure followed by the PowerFood® Microbial DNA Isolation Kit (MO BIO-Qiagen). The DNA extracted from each sample was quantified using a BioSpectrometer® (Eppendorf, Milan, Italy) and then fragmented and tagged with sequencing indexes and adapters using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Shotgun metagenomic sequencing was performed using the NextSeq 500 (Illumina) 2 × 150 bp in paired-end mode. All shotgun metagenomic raw data were deposited as fastq format in the

Sequence Read Archive (SRA) at the NCBI portal and are publicly available under the project named PRJNA1052994.

2.6. Statistical analysis

The pH, colour, VOCs and fatty acids results at different aging times were compared using parametric ANOVA with Tukey's post hoc test. The Consumers' sensory evaluation results were compared by a non-parametric method (*i.e.*, Kruskal-Wallis test). Statistical analyses were performed using GraphPad InStat version 3.0 (GraphPad Software, San Diego, California, USA). The significance threshold was set at $P < 0.05$. The TBC results were compared by two sample Welch *t*-test and a P value < 0.05 was considered statistically significant.

2.7. Bioinformatic analysis

The metagenomic data were analysed on R environment adopting the package rstatix to compare the relative abundances among groups of interest. The packages ggplot 2 and ComplexHeatmap were used to build barplots and heatmaps respectively. The analysis of spoilage bacteria, yeast and moulds, as well as foodborne pathogens was focused on the microorganisms cited within the EFSA scientific opinion on microbiological safety of aged meat (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2023). Sequencing reads were trimmed using AdapterRemoval (Schubert et al., 2016) with the aim of removing sequencing adapters and stretches of low-quality bases. Cleaned reads were then aligned against the corresponding host genome (bosTau9) with bowtie 2 (Langmead & Salzberg, 2012) algorithm. Unmapped reads were taken into account to run the kaiju pipeline (Menzel et al., 2016) adopted to determine the taxonomical abundance (at all the taxonomic level, from domain to species) by comparing metagenomics reads to the nr_euk database. The abundance of each taxon was calculated as relative abundance.

3. Results and discussion

3.1. Quality parameters of dry aged meat

3.1.1. pH measurement

During the different time points investigated, the pH trend was stable, ranging between 5.50 and 5.58 (5.50 at T0, 5.45 at T1, 5.55 at T2 and 5.58 at T3) with a statistically significant difference ($p < 0.01$) occurring between T1 and T3. This result is in accordance with data reported by Colle et al., 2016, observing meat pH values ranging between 5.50 and 5.66 during 35 days of dry-aging. These values support the activation of calpain by releasing Ca^{2+} ions from the sarcoplasmic reticulum and mitochondria when the level of ATP is practically zero. In addition, these pH values play a pivotal role to maintain the activity of other enzymes such as proteases, including cathepsins, caspases and various N- and C-esopeptidases, that could then promote the genesis of bioactive peptides as confirmed in our study and by other authors (Colle et al., 2016; Kim et al., 2022; Álvarez et al., 2021). The pH stability is an important factor, not only from an organoleptic point of view, but also for the microbiological safety. Van Damme et al., 2022 reported an increase in *Listeria monocytogenes* during aging of a loin with a pH higher than 6, while it decreased in the loins with a pH < 6 (Van Damme et al., 2022).

3.1.2. Colorimetric results

Results of the colorimetric analyses are summarized in Table S1 and shown in Fig. 2. No statistically significant differences concerning the brightness (L coordinate) and other colorimetric indices (a*, redness and b*, yellowness) were observed during aging times in Holstein aged meat, thus highlighting the maintenance of the fresh meat colour during all the maturation process even after 60 days. In the present study, all meat samples were trimmed prior the analysis in order to simulate a real

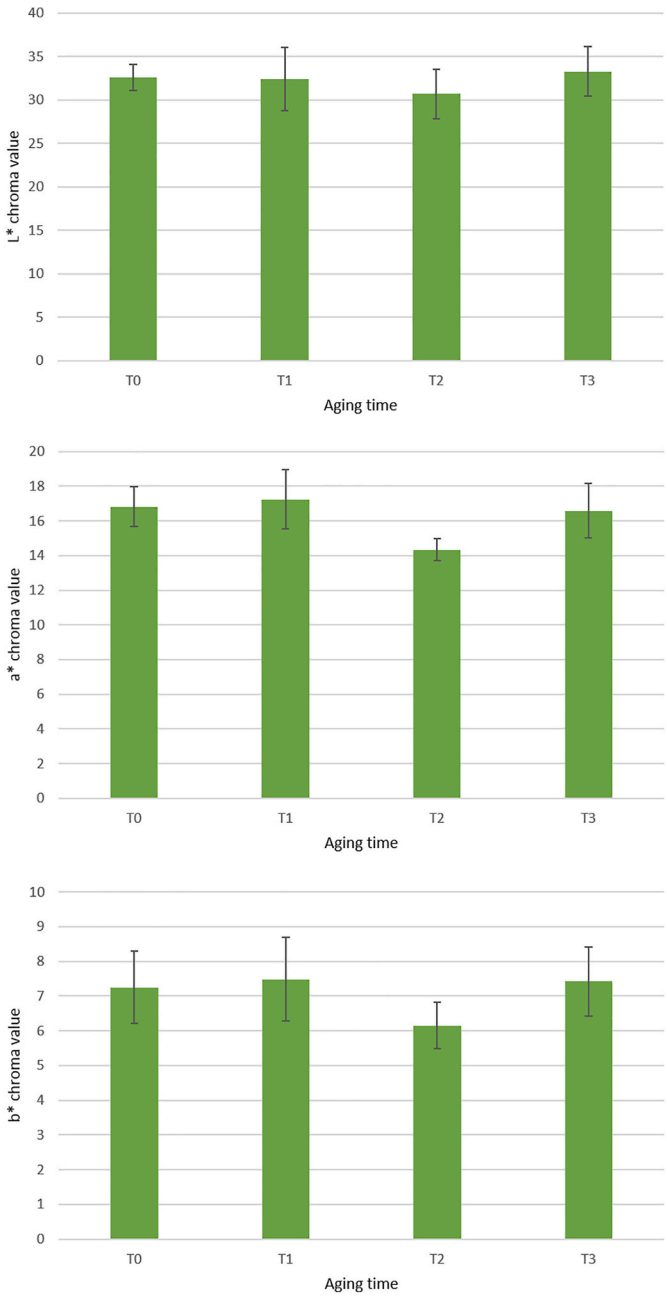

Fig. 2. CIE L*a*b* colour coordinates determined during the aging process (mean and standard deviation); bars indicate standard errors of differences of the means; means with (*) are significantly different ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

scenario in which the external part, characterised by brown colour is usually removed since the consumer could be negatively influenced. The b* value stability here observed during all aging points is crucial during aging times, since it is related to possible oxidation phenomena that could occur during meat shelf life or aging. No meat discoloration as well as fat yellowing were observed during the sensory evaluations.

Lipid oxidation generally causes colour changes modifying white to yellow of meat fat as well as the generation of high aldehydes and ketones content, these last responsible of rancidity of meat and considered as off-flavours when dominant (Kim et al., 2019; Lee et al., 2015). Meat colour represents an important quality parameter in beef because of its influence on the consumer's purchase and consumption decision, as well as an important feature in assessing the degree of meat aging (Faustman

et al., 2010). Our results are similar to those obtained by Kim et al., 2019; Kim et al., 2016 in which the a* and b* values of rump were not significantly affected by aging or the aging method (28 days) but partially different to those available in literature (Colle et al., 2016; Álvarez et al., 2021) in which the brightness coordinates as well as the redness generally tend to be strongly modified during dry aging times even if scarce literature is available on evaluation of colour and other quality traits of long-term dry aging (60 days).

3.1.3. Detected volatile compounds

Overall, more than 90 volatile compounds were detected and quantified in Holstein meat samples and the main ones are presented in Table S2. Scarce literature is available on volatile compounds behaviour in long dry aging processes (i.e., 60 days or more). The concentration of the main volatile compounds, such as aldehydes, ketones, and alcohols, increased during aging time with maximum concentration at 60 days, in accordance with the observations of Lee et al. (2021). These findings are pivotal, since volatiles could be also adopted as possible markers involved in best practice protocols to monitor the dry aging process. Indeed, volatile compounds, depending on their concentrations and thus in relation to their perception threshold, may represent important descriptors of pleasant aromas (flavour) or alterations markers (off--flavours) usually caused by oxidation or fermentation phenomena in meat (Kim et al., 2022). The volatilome profile was determined for the first time in Holstein meat, involved in PDO cheese production system, to evaluate the feasibility to be used for aging process in order to valorise the overall food chain. The attention was dedicated to the volatile compounds involved in off-*flavour* generation and perception as branched-chain aldehydes (2 and 3 methyl butanal), hexanal, 2,3 butanedione (diacetyl), 3-hidroxy-2-butanone (acetoin) as well as branched alcohols, ethanol and free fatty acids like acetic and butanoic that increased significantly after 60 days of aging. These volatile compounds derived from oxidative phenomena as well as fermentation activities (e.g., lactic acid bacteria) are responsible of rancid and acid notes if present at high concentrations.

The detected ketones, in particular 2,3-butanedione and 2-butanone, could have a positive relationship also with the parameters of tenderness and juiciness (Watanabe et al., 2015). Hexanal, and in general aldehydes, are responsible for the nutty flavour characteristic of dry-aged meats as reported by other authors (Flores, 2018; Kim et al., 2019). Aldehydes contribute largely to beef aroma with sweet, floral, salty, and cheesy notes, because they have low odor thresholds (Lee et al., 2021). 2-ethyl furan and 2-pentyl furan were also detected and they play an important role from an aromatic point of view, as their presence is associated with fruity aromatic notes with hints of toast (Li et al., 2021). Volatile compounds' concentrations detected were furthermore associated with high liking score obtained from consumer evaluations at 60 days of aging without *off-flavour* perception as well. In Holstein meat samples, the concentrations of branched aldehydes, hexanal as well as diacetyl and acetoin were found lower in their concentration at 60 days if compared with other studies.

The flavor liking score can be therefore justified to several aldehydes even if involved in lipid oxidation including hexanal, 2-methylbutanal, and nonanal. This scenario was similar to those reported by Frank et al. (2020) and Song et al. (2011). The study of Song et al. (2011) showed that a moderate lipid oxidation is essential for the formation of beef flavor in cooking. However, in the 56-day dry aged beef, the concentration of heptanal and octanal reached a relatively high level, which may have caused a perception of oxidized flavor by consumers and thus reduced the sensory score (Campo et al., 2006). Prolonged aging also promotes the release of free fatty acids, which after reacting with proteins and other precursors can contribute to the aroma and/or taste of aged meat, resulting in a particular flavor. However, such free fatty acids are susceptible to oxidation and the product lifespan due to the development of off-odor and off-flavor can be reduced. Aging regimen should be carefully designed to develop desirable flavour and minimize undesirable notes.

3.1.4. Fatty acid composition

Results of the fatty acid determination are summarized according their main classes SFA, MUFA and PUFA in Fig. 3 and Table S3. No statistically differences were observed among SFA, MUFA and PUFA classes during 60 days of dry aging. The unsaturated proportion of fatty acid was preserved during aging playing a crucial role in *off-flavor* formation as consequence of fatty acid oxidation as well. The results obtained in the present research partially differ from those presented in other research in which changes in fatty acids were observed during dry aging. Kim et al. (2017, 2020) showed that dry aged beef had higher 20:5n-3 fatty acid content, demonstrating that the C20:2 and C20:3n-6 content of beef increased during the aging process. In other studies, Utama et al. (2020) reported the longer the aging period (<40 days), the higher the amount of malondialdehyde and lipid oxidation. The changes in fatty acid composition were observed after 40 days of aging, resulting in an increasing proportion of n-6 polyunsaturated fatty acids and, thus, an increase of the n6 to n3 polyunsaturated fatty acids ratio (Utama et al., 2019). The changes in fatty acid composition were observed from the authors after 40 days of aging, particularly the reducing proportion ($p < 0.05$) of oleic acid (C18:1n9), gamma-linolenic acid (C18:3n6), and alpha-linolenic acid (C18:3n3), and the increasing proportion ($p < 0.05$) of arachidonic acid (C20:4n6). The maintenance of the fatty acid composition represents an important indication toward oxidation phenomena that could promote oxidative changes in the deep parts of the loins and it depends both on meat quality as well as on the aging parameters since temperature, relative humidity and air flow are involved. Since beef flavor is the key for eating quality and overall acceptability (Dashdorj et al., 2015), the increase of fatty acids during aging needs to be controlled as this increase can cause undesirable notes, after taste (Kim et al., 2019).

3.1.5. Peptidomics and proteomics results

Mass spectrometry based peptidomic and proteomic investigations were performed with the aim to gain direct experimental evidences of time-dependent changes caused by proteolytic and oxidation-induced post-translational modifications (PTM) processes undergoing during dry aging. Peptides derived from endogenous proteolysis and PTM of proteins affect digestibility, potential beneficial or harmful effects (production of biologically active peptides or allergenic forms), tenderness and organoleptic characteristics of food.

A statistically significant increase (Student's t-test $P < 0.05$) in peptide number was observed at T1 (768 ± 60) vs T0 (660 ± 104) and T3 (890 ± 12) vs T0 (660 ± 104). Total extracted ion current (TIC) intensity associated to endogenous peptides is statistically different ($P <$

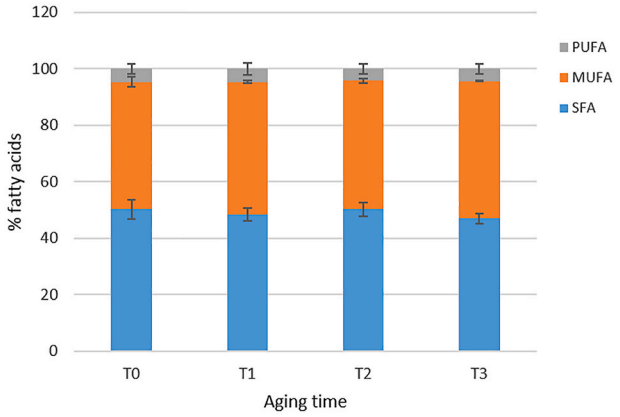

Fig. 3. Fatty acid trend during the aging process (SFA, MUFA and PUFA); bars indicate standard errors of differences of the means; means with (*) are significantly different ($p < 0.05$).

0.05) at T3 (3.43E + 11 8.29E+10) vs T0 (1.54E + 11 4.41E + 10). Direct measurement of one of the proteases known to be responsible for biochemical processes in meat maturation, *i.e.* gelatinase/collagenase, which still shows a specific activity of 0.09 U/mg total proteins (n = 3) at T3, confirms that indeed proteolytic processes are still operative after 60 days. A total of 1066 different peptides deriving from endogenous proteolysis have been detected. As it can be clearly inferred from their sequences, peptides originate from both endogenous endo- and eso-proteases with several different specificities. This, while hampering meaningful conclusions deriving from bioinformatic tools to detect the main protease(s) responsible for the extended proteolytic events observed during aging process under these conditions, indirectly confirms that different proteases with different specificities, including calpains, cathepsins, caspases and various N- and C-esopeptidases, are active throughout the whole period.

Interestingly, progress of proteolysis resulted in an increased number of potential bioactive peptides. Fig. S1 shows that the number of potential bioactive peptides (including precursor peptides which can generate the final bioactive form by action of endogenous amino- or carboxypeptidases) tend to increase at T3 vs T0.

A mass spectrometry-based shotgun proteomic approach was applied to detect possible time-related PTM induced by the dry-aging conditions. We focused on detection of hydroxylated Tyr, Trp, Pro, Lys and on Met sulfoxide or sulfone. The rationale was to detect potential statistically significant increase in the modified form with time. In particular, detection of variation in hydroxylated Tyr, Trp and Met sulfone, often associated with oxidative effects depending on processing/storage conditions, could be very informative. No statistically significant differences in any PTM were detected during time showing that no substantial oxidation of protein residues was undergoing during dry aging. This result is relevant considering that oxidative environments have been shown to decrease tenderization of beef steaks through inactivation of μ-calpain1 (Rowe et al., 2004). All in all, no statistically significant differences in any oxidative-related PTM was detected on time and different proteases, including calpains, cathepsins, caspases and various N- and C-esopeptidases, were active throughout the whole period. The progression of proteolysis resulted in an increased number of potential bioactive peptides.

3.2. Histological evaluations

Histological features at T0 showed any appreciable change in the architecture of muscle, connective and adipose tissue (Fig. 4 A and D). The muscle fibers showed a polygonal or slightly rounded profile: these observations are in line with what was reported by (Kozyrev et al., 2018) in beef after 4 days of maturation.

In the longitudinal section the majority of skeletal muscle fibers showed an irregular pattern accompanied by some fragmentation points (Fig. 4 C). The most significant structural changes were observed starting from 30 days and continuing for the subsequent sampling periods (45, 60 and 90 days). After 30 days of aging (T1) (Fig. 5A–D), we observed an increase in spaces between muscle fibers as well as an increase in fibers with a rounded profile (Fig. 5 A). Similar morphological aspects were described by Kandeepan and Porteen (2016): the authors observed, after 30 days of aging of the beef, an increase in the spacing between the muscle fibers and how the latter, with a rounded profile, are greater in number than those with a normal polygonal profile. These previous observations after 30 days were confirmed by evaluations of the histological preparations in longitudinal section: in these (Fig. 5 B), the fragmentation process of the fibers is much more pronounced than in the previous samples (0 days). In addition, in longitudinal section, fragmented muscle fibers with partial loss of sarcomeric organization were observed (Fig. 5 D): one of the causes of striation impairment appears to be oxidative processes resulting in accelerated/increased with

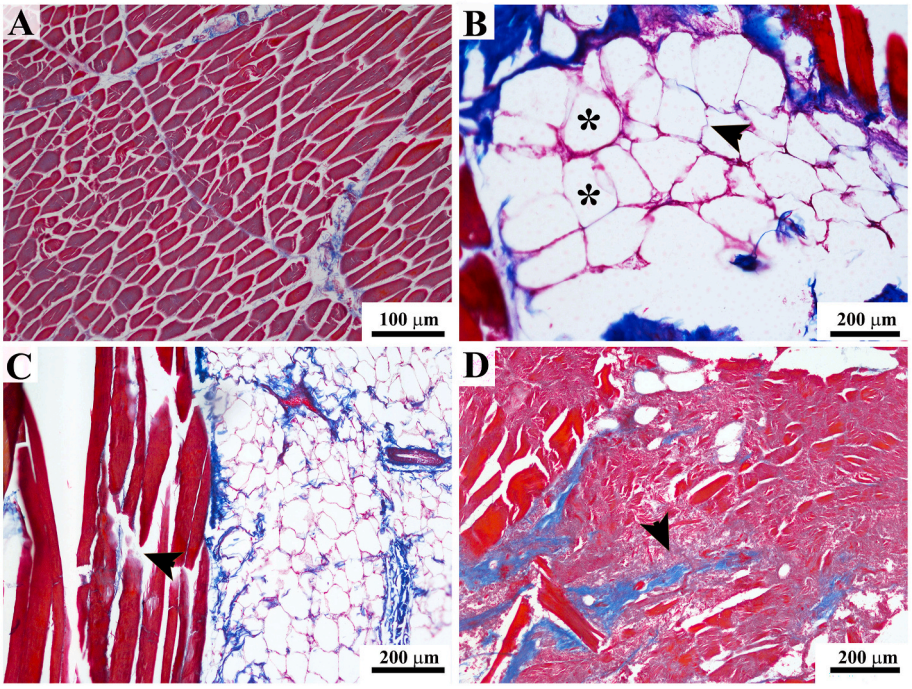

Fig. 4. Histological sections of muscle tissue oriented in both transversal and longitudinal directions, obtained from samples taken from Holstein ribs at time 0 (0 days of aging) stained with Masson's trichrome method (A–D). In image A, the muscle tissue has a normal architecture with muscle fibers having a polygonal profile. In some areas of the section, the peri- and endomysial connective tissue appears degenerated and structurally altered. In addition, this compromised connective tissue shows a change in staining affinity: the classic blue colour of the connective tissue is replaced by a pink-purple colour (D, arrowhead). Adipocytes with a normal rounded profile (B, asterisks) were observed alternating with adipocytes in which the membrane shows an irregular pattern (B, arrowhead). In longitudinal section, the majority of skeletal muscle fibres show an irregular pattern accompanied by fragmentation points: in these fibers, the nuclei are poorly stained (C arrowhead). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

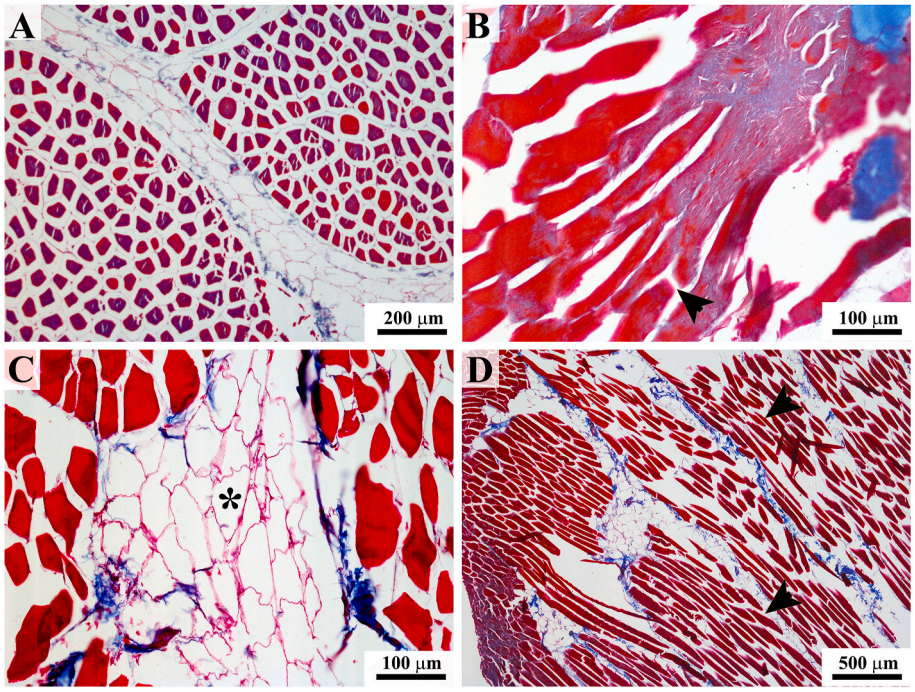

Fig. 5. Representative images of histological sections of muscle tissue, oriented both transversely and longitudinally, obtained from samples taken from Holstein ribs after 30 days of aging, stained using Masson's trichrome (A–D). Most of the muscle fibers are spaced apart and have a rounded profile (A and C). Several fibers show fragmentation (B, arrowhead) and loss of striations. The adipose tissue consists almost entirely of adipocytes with an irregular cell membrane (C, asterisk). In the longitudinal section, the fragmentation at different points along the length of the fibres is even more evident (D, arrowheads).

an improvement in meat tenderness as described by Feng et al. (2020). These oxidative phenomena are not related to off-flavour development as demonstrated in the present research, since are connected to enzymatic activities occurred during all aging points and responsible of peptide generation.

Moreover, after 30 days, the connective tissue was clearly compromised. Collagen is the major connective tissue protein in animals, and it provides the structural framework for muscle and adipose tissue (Flint & Pickering, 1984). In this study we observed, after 30 days of storage and in the successive sampling periods, a progressive compromise of perimysial and endomysial connective tissue (Fig. 5 D). In this regard, in samples of beef preserved for 28 days, (Kozyrev et al., 2018) observed signs of rarefaction and degeneration of the perimysial component and its separation from the muscle fibres. These processes are closely related to the presence, quality and distribution of the connective tissue (especially collagen) and represent one of the factors affecting tenderness in beef (Ledward, 1984; Rhee et al., 2004). In addition, the adipocytes show a marked reduction in size and the nuclei seem to have disappeared (Fig. 5 C).

After 45 days, 60 days and 90 days of storage (Figure S2 A-E), there is no difference in the type of microscopic changes compared to those previously described, although they are more pronounced in some areas in the samples aged for 30 days. In cross-sections, the muscle fibers are still spaced apart and the normal architecture of the muscle tissue is compromised. The majority of muscle fibres exhibit an increasingly rounded profile, and the nuclei disappear. In longitudinal sections, an increase in fragmentation phenomena was observed: the course of the muscle fibres appeared interrupted at several points and several fibers exhibit a wavy/sinusoidal pattern. Again, there is an increase in areas without characteristic striations. The connective tissue between the fibers appears increasingly rarefied/absent, while the adipose tissue is always present between the muscle fibres and the adipocytes (of small dimensions) show an irregular cell membrane.

3.3. Consumers' sensory evaluation results

The sensory evaluations of Holstein meat are reported in Table 1. The flavor descriptors were selected to evaluate the possible generation of *off-flavor* notes considering the 60 days of aging and the fact that the meat was obtained from milk producing animals. The sensory panel indicated high scores in overall liking and olfactory intensity, with an increasing trend ($p < 0.05$) during the aging period, reaching a maximum at 60 days (Table 1). A positive relationship was confirmed between flavor liking and the concentration of furans. This result is in agreement with the finding of Frank et al., 2017, who reported that 2-ethyl furan and 2-penthyl furan are important contributors to the grilled flavor and flavor impact attributes in the aged meat. Similarly, 3-hydroxy-2-butanone, has been reported to positively influence the flavor

Table 1
Consumers' sensory evaluations at different aging times (mean ± s.d.).

	Aging times				P-value*
	T0[a]	T1	T2	T3	
Lean part colour (intensity)	0.20 ± 0.00	0.42 ± 0.29	0.58 ± 0.14	0.67 ± 0.29	0.207
Fat Colour (from white to yellow)	0.47 ± 0.50	0.58 ± 0.38	0.58 ± 0.14	0.58 ± 0.14	0.935
Uniformity of colour	0.13 ± 0.12	0.33 ± 0.29	0.25 ± 0.25	0.25 ± 0.00	0.470
Olfactory intensity	1.33 ± 0.23	2.75 ± 0.25	3.42 ± 0.29	4.58 ± 0.14	***
Off flavour (unpleasant notes)	nr	nr	nr	0.58 ± 0.14	0.063
Acidic smell	nr	nr	0.08 ± 0.14	0.25 ± 0.00	0.235
Rancid smell	nr	nr	nr	0.25 ± 0.00	0.072
Overall liking	7.87 ± 0.23	7.50 ± 0.25	7.58 ± 0.58	8.08 ± 0.29	*

*P < 0.05; **P < 0.01; ***P < 0.001.
[a] Sensory score scale (0 minimum – 9 maximum); nr = not perceived.

profile of cooked and aged meat (Ha et al., 2019). Hence, it is postulated that the higher concentration of furans and 3-hydroxy-2-butanone in dry aged beef contributed to its high flavor liking in sensory assessment of beef and fat. In our study the concentration of 3-hydroxy-2-butanone was positively related to the flavour liking. A similar finding was reported by Li et al., 2021 and Bulgaru et al., 2022. Flavor liking score was also positively influencing several aldehydes produced by lipid oxidation, including hexanal, 2 and 3-methy butanal and nonanal, since no rancid notes were perceived in the present study. The consumer evaluation of Holstein aged meat showed that the presence of some volatile compounds derived from lipid oxidation were associated to positive flavor of aged meat also confirmed by volatile compounds profiling as well as fatty acids determinations in which the absence of oxidative phenomena during 60 days of dry aging was demonstrated.

3.4. Microbiological safety parameters

3.4.1. Enumeration by culture methods

In the Holstein surface samples, the mean TBC count at T0 was 2.81 ± 0.57 Log_{10} CFU/g and significantly increased at T2 (4.35 ± 0.68 Log_{10} CFU/g, P = 0.042) and at the end of the aging period (6.23 ± 0.97 Log_{10} CFU/g, P = 0.01). The TBC mean Log_{10} value at T0 is mostly related to the microbiological status of the animal of origin (Botta et al., 2023), the contamination during slaughtering and following manipulations and/or the storage conditions before dry aging. Metagenomic analysis revealed that LAB along with *Pseudomonas* appeared to be the most prevalent microorganisms found in the samples analysed. The mean log TBC reported in our study at T0 were in line with those reported by (Hulánková et al., 2018) and by (Khazzar et al., 2023) that recorded 2.59 ± 0.65 log_{10} CFU/cm^2 on fresh meat, and 2.5 log_{10} CFU/cm^2 after a seven days carcass storage period. In contrast, our data were lower than what described by (Di Paolo et al., 2023) that enumerated 4.10 log CFU/g after 2 days of aging.

In the meat the mean TBC increased during the first 30 days, in line with what reported by other authors (Di Paolo et al., 2023; Khazzar et al., 2023; Lee et al., 2017). After T1, bacterial counts remained stable through time in line with Ryu (Campbell et al., 2001; Hulánková et al., 2018; Ryu et al., 2018) reported an increase of TBC during the first week of ageing, followed by a period, up to 21 days of ageing, in which the TBC remained substantially unchanged. Smaldone and colleagues on the contrary reported a slight decrease of the TBC throughout 290 days of maturation starting from 6.82 Log_{10} CFU/g at 13 days after slaughter and 6.13 Log_{10} CFU/g at 290 days of maturation.

The Enterobacteriaceae counts in all samples were beneath the limit of quantification of the analytical method (i.e., 10 CFU/g). Our results are in line with the data obtained by (Gowda et al., 2022), that reported a median Enterobacteriaceae count below the limit of quantification of the method in samples collected from 15 dry-aged meat producing companies in Belgium. Besides, when present, Enterobacteriaceae are reported to decrease (Li et al., 2013; Smaldone et al., 2019) but also to increase (Li et al., 2014) during time. In the latter study it should be noted that meat was aged in a refrigeration room without control of humidity and air flow. All in all, the divergencies among our results and other studies might be partly attributed to the process parameters set within the dry aging cabinets used in the different studies, but also to the fact that during maturation the population differences have been demonstrated to be based on the aging's facility's microbes and environmental conditions (Capouya et al., 2020).

3.4.2. Shotgun metagenomic analysis

At each sampling time the surface samples were also tested by shotgun metagenomics and the results revealed that the microbiome was heterogenous among samples collected during the aging period. The taxonomic analysis showed that Bacteria was the prevalent domain but Eukariota were also represented, including fungi (Opisthokonta) and SAR (Stramenopiles, Alveolata, Rhizaria). At the phylum level, the taxonomic analysis showed the presence of Proteobacteria and Firmicutes as prevalent phyla (Fig. 6 A), in line with the findings of (Umiralieva et al., 2021), that reported the same phyla in the microflora of refrigerating chambers, with the addition of *Actinobacteria,* that we also detected. The phylum Proteobacteria became prevalent after 60 days of aging and this is in partial agreement with the results from (Ryu et al., 2020), that reported Firmicutes to be the most dominant in all time period, with a reduction by aging of the relative abundance and an increase of the proportion of Actinobacteria and Proteobacteria at 70 days of ageing. In Holstein loins the percentage of Proteobacteria decreased during the final phase (90 days) in favor of *Mucormycota* despite the absence of evident moldiness.

A more complex composition was evidenced at the genus level (Fig. 6 B), with *Pseudomonas* being the most represented genus, followed by *Photobacterium*, *Staphylococcus* and *Thamnidium*. In the Holstein surface samples, despite a greater heterogeneity of the initial microbiome composition, *Pseudomonas* tended to be more represented as the maturation proceeded in agreement with Ryu et al. (2020), in association with a higher amount of *Thamnidium* after 90 days. Similarly, (Capouya et al., 2020) described the presence of a high proportion of reads belonging to *Pseudomonas* in meat samples collected in five commercial dry aging facilities. Despite the high prevalence in the surface samples of *Pseudomonas* spp. and *Photobacterium*, considered common meat spoilers (Fuertes-Perez et al., 2019; Nychas et al., 2008) we did not evidence spoilage phenomena along the ripening period.

Focusing on spoilage bacteria, they were mostly represented by *Pseudomonas* and Lactic Acid Bacteria (LAB) including *Streptococcus, Enterococcus* and *Facklamia*. All spoilage microorganisms decreased during the dry aging process, except *Pseudomonas* and *Brochotrix* that increased on time. A reduction of LAB during ageing was previously observed by Ryu et al. (2020).

Regarding yeasts and moulds (Fig. 7), over the course of the 90-days maturation period, we were unable to observe any change in the relative percentages of *Aspergillus*, and *Penicillium* under the maturation conditions used. *Aspergillus* and *Penicillium* are capable to produce mycotoxins, all-be-it the formation of mycotoxins below 5 °C is considered very unlikely (Olivier, 2018). For the Holstein surface samples a wavy trend was registered with a higher contamination at T1 with different species (namely *Thamnidium*, *Mucor* and *Rhizopus*), followed by a decrease of the whole reads through T2 and T3, and an increase at T4. *Thamnidium*, *Mucor* and *Rhizopus* and *Debaryomyces* resulted the most represented moulds in Holstein surface samples during all the maturation period in partial agreement to Capouya et al., (2020) and Ryu et al. (2020).

In relation to the presence of reads of foodborne pathogens, a relative abundance above 1% in at least one sample was evidenced only for *Staphylococcus aureus* and *Campylobacter coli*, while for other pathogens such as *Salmonella enterica*, *Listeria monocytogenes* and *Clostridium botulinum*, the detected reads were very few. Based on the predicted growth scenario from EFSA Panel on Biological Hazards (BIOHAZ) et al. (2023) the main microbiological hazard to consider in the meat dry aged is *L. monocytogenes*.

4. Conclusions

In this study we investigated the quality parameters and microbiological safety of Holstein loins dry aged for 90 days. The number of tested loins was limited due to the difficulty to perform the study with more samples in experimental conditions. However, many parameters were measured at five different sampling times. Moreover, the microbiological evaluations by classical culture methods have been complemented by microbiome results.

No substantial oxidation of protein residues was detected during the dry aging process investigated in our study. This result is very relevant because oxidative environments have been shown to decrease tenderization of beef steaks through inactivation of μ-calpain1. The endogenous

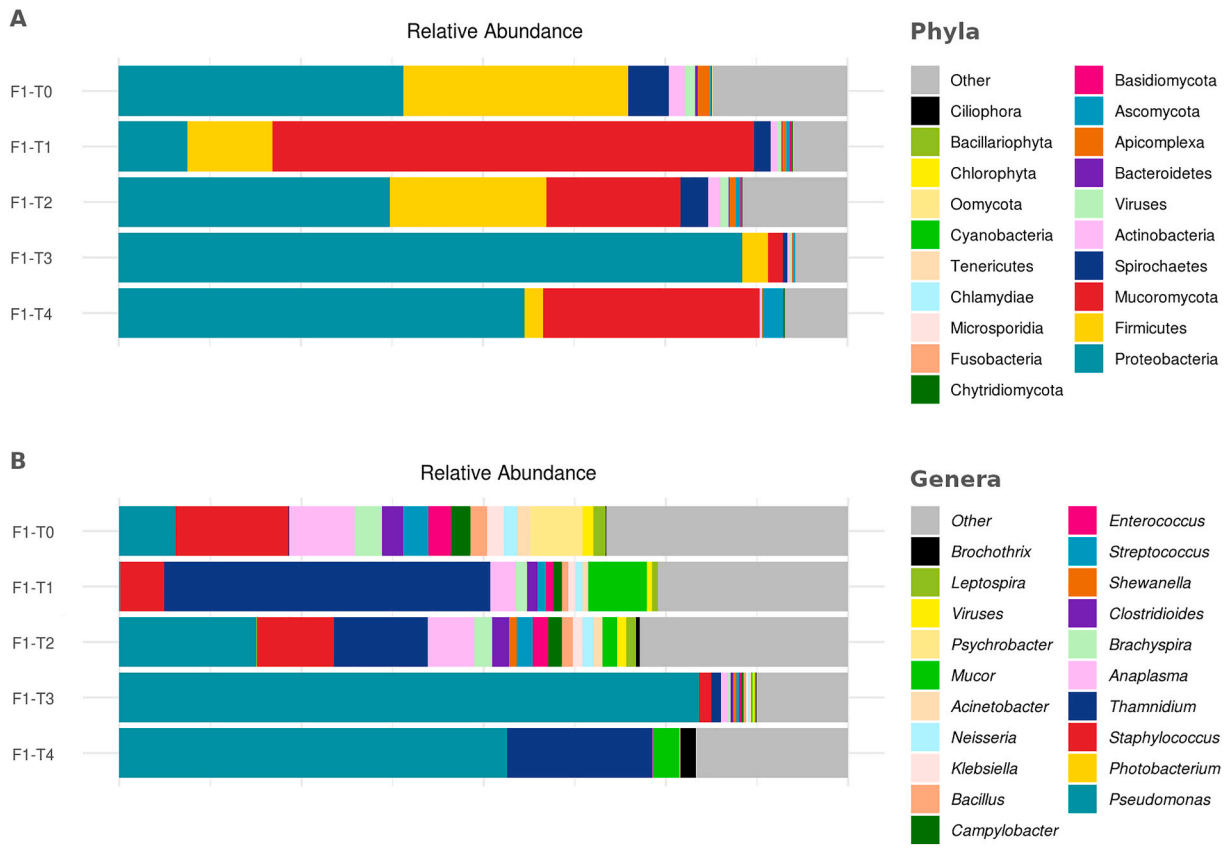

Fig. 6. Top 20 phyla (A) and genera (B) detected in the Holstein surface samples during the aging period.

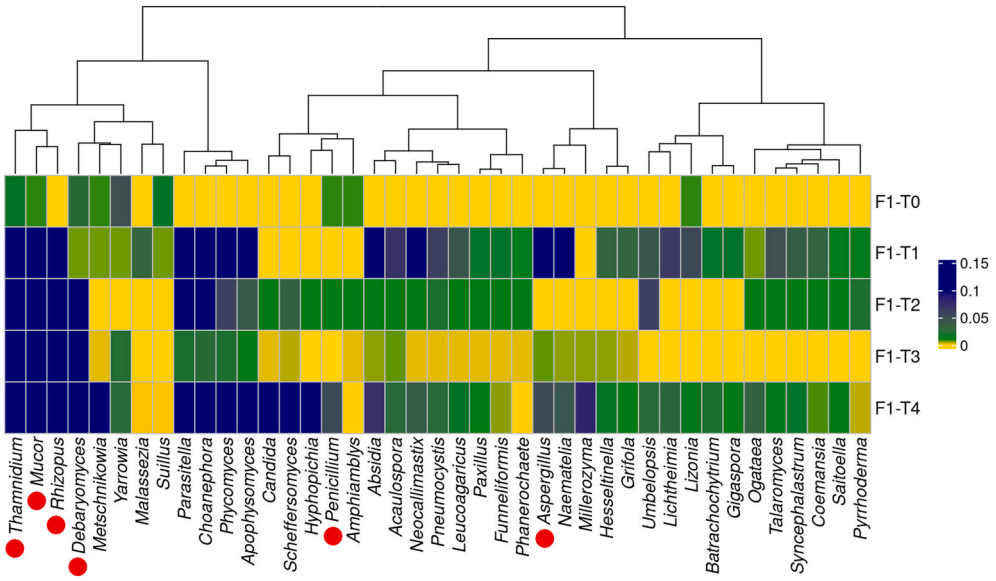

Fig. 7. Heatmap representing the distribution of the fungal component in Holstein samples. Red dots identify fungal genera cited in the EFSA Panel on Biological Hazards (BIOHAZ) et al. (2023) opinion on dry aged meat. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

generation of bioactive peptides in beef through postmortem aging was demonstrated. Peptides derived from endogenous proteolysis and PTM of proteins affect digestibility, potential beneficial or harmful effects (*e.g.*, production of biologically active peptides or allergenic forms), tenderness and organoleptic characteristics of meat. The main proteins undergoing proteolytic degradation, were myoglobin, myofibrillar constituents and sarcoplasmic enzymes, including creatine kinase and glycolytic enzymes. Interestingly, progress of proteolysis resulted in an increased number of potential bioactive peptides. While, as for all bioactive peptides in food (including milk casein derived peptides) the possibility for such peptides to reach blood, escaping gastrointestinal digestion, is still a matter of debate, it is however interesting to note that

in our study we discovered that the peptides produced through the dry aging process showed potential biological activities, such as enzymatic inhibition, including ACE inhibition, antihypertensive, antioxidative, antibacterial and opioid properties (Table S3).

The histological results showed structural changes in the skeletal muscle fibers starting from 30 days. However, any significant change in the structure of the muscle was observed during the aging period. Although an expert (morphologist) can easily distinguish dry aged meat from fresh meat, from a legislative point of view the results of our study cannot define that the cut surface shows that the product no longer has the characteristics of fresh meat according to Regulation EC 853/2004. Therefore, from a legislative point of view, more elements are needed to evaluate the classification of dry aged meat in the category of meat products.

The results on microbiological safety parameters showed that total bacterial count increased during ageing but without signs of spoilage in the inner part of the meat. Few reads of *L. monocytogenes* were detected and the only pathogens for which a relative abundance >1% was quantified were *Staphylococcus aureus* and *Campylobacter coli*. However, *Staphylococcus aureus* and *C. coli* are not considered of primary importance in dry aged meat (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2023), as they are not able to grow and/or produce toxins at the temperatures used for dry ageing. While no data are available in literature concerning these pathogens in dry aged meat, in our study we observed a reduction of the relative abundance of their reads during the ageing process. These results contribute to advance the knowledge on presence and behavior of foodborne pathogens in dry aged meat. They can be used by food producers to assess the possibility to extend the meat dry aging process when the latter is performed under strict production conditions. Moreover, they can be useful for food control authorities to define microbiological criteria to validate the production parameters of dry aged meat.

The metagenomic analysis highlighted the changes in the meat microbiome during ageing but, as highlighted by Capuoya et al., (2020), as the maturation proceeds the microbial community tends to become more homogeneous, with *Pseudomonas* spp. replacing the other microbial populations. To our knowledge, two previous metagenomic studies were published on dry aged meat (Capouya et al., 2020; Ryu et al., 2020) with both *Pseudomonas* spp. and *Mucor* spp. found to be prevalent, suggesting a common core microbiome for dry-aged beef as previously speculated by Ryu et al. (2020).

In conclusion, the results of this study provide the first insights on the quality parameters and microbiological safety of dry aged Holstein loins. More data are needed on dry aged meat obtained in commercial conditions and in different production settings to build decision making tools supporting food companies to improve both quality and safety of dry aged meat products. The application of dry aging to meat can help to reduce food waste and produce innovative products. Moreover, as far as Holstein meat is concerned, its valorisation in the emerging market of dry aged meat would certainly enhance the economic, social and environmental sustainability of the Parmigiano Reggiano designation of origin food system.

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CRediT authorship contribution statement

Federica Savini: Writing – original draft, Methodology, Investigation, Data curation. **Valentina Indio:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Sara Panseri:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Luca Chiesa:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Armando Negri:** Writing – original draft, Investigation, Data curation. **Francesca Grassi Scalvini:** Writing – original draft, Investigation, Data curation. **Alessandra De Cesare:** Writing – original draft, Data curation. **Maurizio Mazzoni:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Paolo Clavenzani:** Writing – review & editing. **Laura Prandini:** Writing – review & editing, Investigation. **Federico Tomasello:** Investigation, Data curation. **Yitagele Terefe Mekonnen:** Investigation. **Federica Giacometti:** Investigation. **Andrea Serraino:** Writing – review & editing, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

I have shared the information about data into the cover letter

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116390.

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