The Microbe 2 (2024) 100035

Contents lists available at ScienceDirect

The Microbe

journal homepage: www.sciencedirect.com/journal/the-microbe

Comparison of bacterial diversity in wet- and dry-aged beef using traditional microbiology and next generation sequencing

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ARTICLE INFO

Keywords:
Dry-aged
Wet-aged
Meat microbiota
Next-generation sequencing

ABSTRACT

The science behind dry-aged meat has advanced majorly during the last few years. Unlike wet-aging, where meat is vacuum packed, the dry-aging process is conducted without packaging or protection, which may change its bacterial diversity and consequently alter its sensory characteristics. Traditional techniques and Next Generation Sequencing (NGS) stand out among the different methods used to identify bacterial diversity. This study evaluated the bacterial diversity of dry- and wet-aged beef using traditional microbiological tests and NGS to compare their specificity in bacterial diversity identification. Samples from beef strip loins (n = 6) were collected directly from the slaughterhouse and transported to the laboratory, where they were dry- or wet-aged for 20 and 34 days. Before and after aging, the samples were analyzed by traditional microbiological testing and NGS. Traditional microbiology testing found an increase in total bacterial count, particularly of psychrotrophic bacteria, in the wet-aged samples from 0 to 20 and 34 days. Dry-aged samples showed a decrease in the total bacterial count, with only molds and yeast presenting significant growth during aging. Metagenomics analysis detected eleven main bacterial genera in the meat microbiota, with a relative abundance higher than 2 %, including Carnobacterium, *Pseudomonas*, *Lactobacillus*, *Romboutsia*, *Leuconostoc*, *Candidatus Nitrosotalea*, and *Akkermansia*. Alpha diversity showed a higher richness in non-aged samples, whereas wet-aged samples (20 and 34 days) showed the lowest richness. Moreover, beta diversity analysis found that the microorganisms are highly related when considering time but form different clustering when comparing the aging process. Dry-aged beef had a higher presence (80.9 % on the 34th day) of *Pseudomonas* sp., a group of microorganisms with a large range of ideal bacterial growth conditions. Conversely, due to their controlled anaerobic environment, wet-aged samples showed a higher presence (79.4 % on the 34th day) of *Carnobacterium*. Traditional microbiology testing remains an important tool to ensure food safety since it can clearly identify the main groups of bacteria present in food. NGS, in turn, allows to identify more microbial groups but is an expensive tool, especially when considering the number of samples. Despite showing different data specificity, both techniques efficiently differentiated the beef microbiota.

1. Introduction

Meat is a staple in the human diet since it contains several essential nutrients (lipids and proteins of high biological value) and micronutrients such as iron, zinc, and vitamin B12 (You et al., 2022). Such high nutrient content, coupled with the influence of environmental

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https://doi.org/10.1016/j.microb.2024.100035
Received 26 September 2023; Received in revised form 23 December 2023; Accepted 8 January 2024
Available online 11 January 2024

factors, such as temperature, atmospheric oxygen, endogenous enzymes, moisture, and light; makes meat an excellent substrate for numerous microorganism species to penetrate, grow, and multiply (Barcenilla et al., 2022; van Reckem et al., 2021).

Studies indicate that meat, even from healthy animals, can encounter contamination from the bleeding process to commercialization; with the types of spoilage microorganisms being influenced by storage conditions (Terjung et al., 2021; Tyuftin and Kerry, 2023). The role of bacterial species in meat spoilage is well established (Zagorec and Champomier-Vergès, 2023), and the microorganisms found on the meat surface can impact the quality and effectiveness of the aging process (Terjung et al., 2021). Aged meat microbiota may contain lactic acid bacteria (LAB), and mesophilic and psychrotrophic bacteria; and when in large quantities, force the exclusion of the contaminated meat from sale, causing economic losses to producers and consumers (Xu et al., 2022).

The tenderness, juiciness, and flavor of meat are influenced by factors that include breed, animal age, feed type, breeding, and aging time (López-Pedrouso et al., 2020; Salzano et al., 2021). In addition to its preservation benefits, the aging process has garnered significant attention in the past decade for its capacity to enhance the sensorial aspects of meat. This has led to numerous studies exploring the transformative effects of aging on meat quality. (Valoppi et al., 2021). Two aging processes are used to cater to the preferences of the consumer: wet- or dry-aging processes (Terjung et al., 2021; Shi et al., 2020) Notably, both wet-age and dry-age have been found to elevate the quality of diverse beef cuts, indicating the potential for pursuing an optimal method-time and aging combination. This suggests that a careful balance of factors can be tailored to meet consumers' preferences and achieve desired beef characteristics (Salzano et al., 2021; Lopez-Caballero et al., 2001).

However, factors such as temperature, relative humidity, exposure to forced air, and the group and quantity of microorganisms on the meat surface can interfere with meat quality and yield during aging (Oh et al., 2019; Di Paolo et al., 2023). More common due to the production yield and convenience of storage and transportation (Dashdorj et al., 2016), wet-aging involves sealing meat in vacuum packages and storage in refrigerated temperatures (between −1 °C and 2 °C) for a determined period (Terjung et al., 2021). Dry-aging refers to unpackaged meat cuts kept on open racks in a temperature and humidity (Terjung et al., 2021). Despite possible losses due to evaporation, crust formation, risk of contamination by microorganisms during the process, and the space and materials required (Terjung et al., 2021; Ryu et al., 2018), consumers are willing to pay for this expensive product because of its quality and flavor (Dashdorj et al., 2016).

Since the effects on meat microbiota can be aging method-specific (Terjung et al., 2021), bacterial diversity should be evaluated. Next Generation Sequencing (NGS) is a broadly used technique to study the bacterial composition of ecosystems, delivering more precise results regarding bacterial diversity (van Reckem et al., 2021; Di Paolo et al., 2023). Thus far, few studies have compared traditional microbiology tests with NGS in meat products. Therefore, this study aims to evaluate the bacterial diversity of dry- and wet-aged beef produced in Brazil by Next Generation Sequencing of the 16 S (rRNA) gene and by traditional microbiology testing; comparing their results to understand the diversity with respect to each aging process.

2. Material and methods

2.1. Origin, sample collection, and meat aging

The experiment utilized 60-cm beef strip loins (n = 6) sourced from ½ Nellore x Angus young steers. Initial fresh (unaged) meat samples were collected on day 0 (n = 6) for baseline characterization. Each strip loin was halved, with one portion assigned to wet treatment and the other to dry treatment. After 20 days of aging, both sections were sampled (n = 6 for each treatment) and returned for additional aging. A second round of sampling occurred at 34 days of aging (n = 6 for each treatment).

On day 20, vacuum-packed samples were aseptically extracted from the packaging, and fragments from the meat surface (~100 g) were removed before repacking. The surface crust (~100 g) was aseptically removed for the dry-aged sections. Following sampling, all cuts were returned to the aging-chamber for continued aging. At 34 days of aging, a second round of sampling was conducted.

The aging process took place in an aging-chamber set at 2 °C and with a relative humidity of 70 %.

2.2. Traditional microbiological analysis

A total of 30 samples were analyzed, where 6 samples were from non-treated meat, 12 from the dry treatment, and 12 from the wet treatment. Six samples were collected at Day 0 (control), and 12 samples from Day 20, and Day 34 (wet- and dry-aged). Twenty-five grams of each meat surface was homogenized in 225 mL of peptone water followed by dilution using Peptone water; serial dilutions were made up t.

The total mesophilic count was conducted using 1 mL of each dilution on Plate Count Agar (PCA) plates, which were subsequently incubated at 35 °C for 48 h. Psychrotrophs were assessed by inoculating 0.1 mL of each dilution and incubating them at 10 °C for 15 days (APHA, 2001).

Enterobacteriaceae testing was performed on Red Violet Bile Agar with Glucose (VRBG) plates incubated at 35 °C for 18–24 h (APHA, 2001). Serial dilutions were placed on MacConkey agar plates and incubated at 37 °C for 24 h (APHA, 2001).

LAB testing was performed using the MRS agar plating method. For each dilution, 1 mL of each sample was inoculated onto overlay MRS agar plates, which were then incubated at 35 °C for 72 h (APHA, 2001).

Yeasts and molds were identified by adding 1 mL of each sample onto Potato Dextrose Agar (PDA) plates and incubated at 25 °C for 72 h (adapted from (APHA, 2001)).

For all tests, plates with 25 to 250 colonies were used for the total bacterial count, and which were then selected and used for further tests (APHA, 2001). The number of colonies counted was multiplied by 10 (per plate) and by the inverse dilution factor of the respective plate. Results were expressed in CFUs/g.

2.2.1. Statistical analysis

Data from the traditional microbiological tests were first evaluated for normality using Shapiro-Wilk and Levene's test ($p \leq 0.05$). Non-normally distributed data were examined using Kruskal Wallis and post hoc analysis, with a 5 % significance level. All statistical analyses were performed using OriginPro software, version 2019b (OriginLab Corporation, Northampton, MA, USA).

2.3. Metagenomics analysis

2.3.1. DNA extraction and sequencing

According to the Bacteriological Analytical Manual, each experimental unit contained 15 ± 1 g of all meat samples (n = 30) mixed with 135 mL of Butterfield Phosphate buffer solution, pH = 7.2 (Food, 1998).

Subsequently, 5 mL aliquots were collected and centrifuged at 13,000 rpm for 10 min at room temperature. After discarding the supernatant, total DNA was extracted from the pellet using PowerFood® Microbial DNA Isolation Kit (Mobio) per the manufacturer's recommendations (da Silva Abreu et al., 2021).

The extracted DNA was then stored at − 20 °C and later sent to the Novogene company for 16 S rRNA gene amplification and sequencing by the Illumina platform. The Illumina MiSeq is a compact and versatile DNA sequencing system used for various applications, such as targeted resequencing and amplicon sequencing. It's valued for its user-friendliness, speed, and production of high-quality sequencing data (Ravi et al., 2018).

2.3.2. Data processing and taxonomic identification

After demultiplexing, quality assessment of paired-end reads from the V4 region of the 16 S rRNA gene sequence was conducted. Following the procedures outlined by (Food, 1998), barcodes were removed, and reads were merged using the SeqPrep C+ + package. Subsequently, quality-filtered reads were unified into a FASTA file and aligned against the SILVA v. 132 database, for closed-reference identification and quantification of Operational Taxonomic Units (OTUs), with a 97 % cluster identity threshold using Qiime 1.9 software (Quast et al., 2012; Yilmaz et al., 2014; Glöckner et al., 2017). Taxonomic classification from phylum to genus level was achieved by referencing a pre-established sequence map for each taxonomic group. The quantified OTUs were consolidated into an OTU table, excluding those with a collective count of fewer than 10 in a minimum of two samples and samples containing fewer than 500 reads. Prior to downstream analysis, OTU counts were normalized to uneven sequencing depth using cumulative sum scaling (CSS) (Paulson et al., 2013). The analysis command lines were adapted from (Biscarini et al., 2018).

All counts and indexes obtained in the previous steps (except the core meat microbiota evaluation) were corrected to baseline before statistical analysis, considering the samples belonging to T0 (unaged group) as a homogenous group. Alpha diversity indices were computed utilizing the R environment (RStudio Team, 2022), with the support of the R packages phyloseq and metagenomeSeq.

2.3.3. Alpha and beta diversity indices

The meat microbial diversity assessment was conducted on two levels: within individual samples (referred to as alpha diversity) and across all samples (referred to as beta diversity). In addition to calculating the observed OTU count directly from the OTU table, within-sample microbial richness and diversity was estimated using Chao1 and ACE (Abundance-based Coverage Estimator) for richness evaluation; the Shannon, Simpson, and Fisher's alpha indices for diversity analysis (Paulson et al., 2013; Biscarini et al., 2018; RStudio Team, 2022; Chao, 1984; Chao and Lee, 1989; Chao and Yang, 1993; Fisher et al., 1943; Shannon, 1948); and Simpson E and Pielou's J (Shannon's evenness) for evenness (Smith and Wilson, 1996). Across-sample diversity was estimated by calculating unweighted UniFrac distances (Lozupone et al., 2011). Between groups (dry-aged, wet-aged, and control), pairwise distances were assessed nonparametrically using permutational analysis of variance [999 permutations] (Anderson, 2001). Details on the alpha- and beta-diversity calculations can be found in (Glöckner et al., 2017).

2.3.4. Statistical analysis

OTU counts underwent one-way ANOVA with the following linear model:

$$y_{ikj} = \mu + T_k + C_j + e_{ikj} \quad (1)$$

Whereas diversity indices were evaluated by one-way ANOVA using two linear models:

$$y_{ikj} = \mu + T_k + e_{ikj} \quad (2)$$

$$y_{ikj} = \mu + C_j + e_{ikj} \quad (3)$$

in which y_{ikj} is the individual value (OTU count or alpha/beta index) for sample i at time point k and treatment j; T_k is the effect of the categorical variable 'time point' (3 classes); C_j is the effect of categorical variable 'treatment' (2 classes); e_{ikj} are the residuals of the model.

3. Results

3.1. Microbiological analysis

Comparisons were performed to evaluate the behavior of microorganisms in different treatments (Table 1). For mesophilic bacteria, an increase in counts for the wet-aged treatment at 20 and 34 days was observed, compared to non-aged samples ($P < 0.05$). No difference in mesophilic counts was found between fresh samples and those dry-aged for 20 and 34 days ($P < 0.05$). When comparing the 34-day-aged samples, higher counts were observed in wet-aged samples compared to dry-aged ones ($P < 0.05$).

Similar to mesophilic bacteria, psychrotrophic bacteria also showed an increase in counts for wet-aged samples aged for 20 and 34 days, compared to non-aged samples ($P < 0.05$). For dry-aged samples, a decline in mesophilic counts was observed when meat was aged for 34 days, compared to non-aged meat ($P < 0.05$). When comparing the 34-day aged samples, higher psychrotrophic counts were observed in wet-aged samples compared to dry-aged ones ($P < 0.05$).

Lactic acid bacteria counts were also similar to mesophilic and psychrotrophic bacteria, an increase in counts was observed in wet-aged samples aged for 20 and 34 days compared to non-aged samples ($P < 0.05$). No difference in lactic acid bacteria counts was found between fresh samples and those dry-aged for 20 and 34 days ($P > 0.05$). No difference between aging times was observed for a given type of aging ($P > 0.05$).

No effect of aging type and time was found for enterobacteria counts, neither for molds nor yeasts ($P > 0.05$).

3.2. Metagenomics analysis

3.2.1. Sequencing metrics

Sequencing of the V3-V4 (Simpson, 1949) regions of the bacterial 16 S rRNA gene from the 16 meat samples yielded 486,823 assembled reads (paired-end R1-R2 spliced reads). After quality filtering, we removed 144,227 sequences, leaving 342,596 sequences for subsequent analysis. On average, 21,155 (± 11,965) sequences per sample in the unaged group were found, 19,717 (± 7152) in the dry-aged group, and 22,975 (± 5455) in the wet-aged group. Initially, 3088 OTUs were identified; after filtering out OTUs with less than ten counts in at least two samples, 379 distinct OTUs remained.

3.2.2. Core meat microbiota evaluation

We identified 11 major bacterial genera from the core meat microbiota, with a relative abundance greater than 2 % (Fig. 1) for the following groups: *Carnobacterium* (47.9 %), *Pseudomonas* (22.2 %), *Lactobacillus* (5.4 %), *Romboutsia* (2.8 %), *Leuconostoc* (2.5 %), *Candidatus Nitrosotalea* (2.4 %) and *Akkermansia* (2.3 %). Genera with an abundance of less than 2 % (125 genera) are not shown.

3.2.3. Differentially abundant taxa

Groups were analyzed for their genus composition, considering an abundance greater than 2 % (Fig. 1).

In comparing the *in natura* samples with the dry-aged meat (20 days),

Table 1
Mean (log CFU/mL) ± SD of microbial counts in different types and aging times.

Aging type	*In natura*	Dry-aged		Wet-aged	
Aging time	Day 1	Day 20	Day 34	Day 20	Day 34
Mesophilic	5.7 ± 0.3^{b}	6.3 ± 0.9^{ab}	5.3 ± 1.3^{b}	7.1 ± 0.7^{a}	7.1 ± 0.3^{a}
Psychrotrophic	5.6 ± 0.5^{c}	6.5 ± 1.1^{bc}	4.1 ± 0.5^{d}	7.5 ± 0.3^{ab}	7.6 ± 0.3^{a}
Latic acid bacterial	5.4 ± 0.9^{b}	6.2 ± 0.6^{ab}	5.6 ± 0.6^{ab}	6.5 ± 0.1^{a}	6.6 ± 0.2^{a}
Enterobacteriaceae	4.6 ± 1.2^{a}	4.2 ± 0.4^{a}	3.7 ± 0.4^{a}	4.8 ± 1.1^{a}	3.9 ± 0.9^{a}
Molds and Yeasts	< 2.0^{a}	2.5 ± 0.6^{a}	2.8 ± 0.7^{a}	2.4 ± 0.2^{a}	2.4 ± 0.6^{a}

Different lowercase letters in the same row indicate significant differences between the treatment by Tukey test ($P < 0.05$).

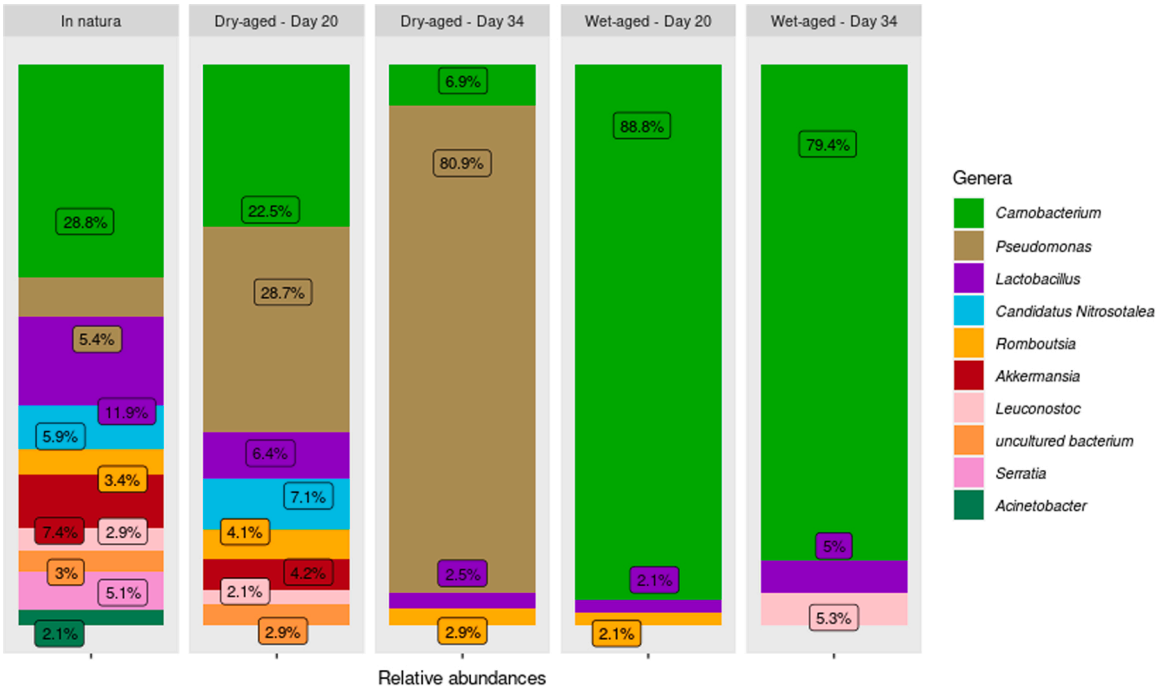

Fig. 1. Microbiome composition of unaged and aged samples, considering an abundance more significant than 2 %.

no significant change in *Carnobacterium* abundance was observed. In contrast, Pseudomonas and *Lactobacilli* were, respectively, more and less abundant in dry-aged samples. The within-sample evaluation found an increase in *Pseudomonas* abundance and a decrease in *Carnobacterium*, *Candidatus Nitrosotalea, Akkermansia,* and *Leuconostoc* for samples dry-aged for 20 days, compared with those aged for 34 days.

Wet-aged samples (20 days) showed a greater abundance of *Carnobacterium* and a lower abundance of *Lactobacillus* and *Romboustia*, when compared with unaged samples; however, *Candidatus Nitrosotalea, Akkermansia, Serratia, Acinetobacter,* and *Leuconostoc* were not identified. Similarly, when comparing the *in natura* samples with those wet-aged for 34 days, we observed a slight decrease in *Carnobacterium*, but it still represented more than 50 % of the entire bacterial count. The abundance of *Leuconostoc* and *Lactobacillus* counts increased in the wet-aged samples.

The traditional microbiological analysis found increased LAB counts in wet-aged samples. Comparison between traditional analysis and bioinformatics confirmed this LAB count increase, which was identified as *Carnobacterium*.

3.3. Alpha and beta diversity

3.3.1. Alpha diversity

Fig. 2 shows the alpha diversity indices estimated to describe the richness, diversity, and consistency of the meat microbiota of the two treatments.

Results of the linear model in Eq. (2) indicate a clear difference in the distribution of p-values for the samples aged for 20 days compared to those aged for 34 days (3 indices – ACE, Chao1, Simpson had p-values less than 0.05; Fig. 2A). Conversely, both aging processes showed a similar distribution in significance (according to the linear model in Eq. (3)), with two indices each having $P < 0.05$ (Chao1 and ACE for dry-aging, and Simpson and Shannon for wet-aging) (Fig. 2B).

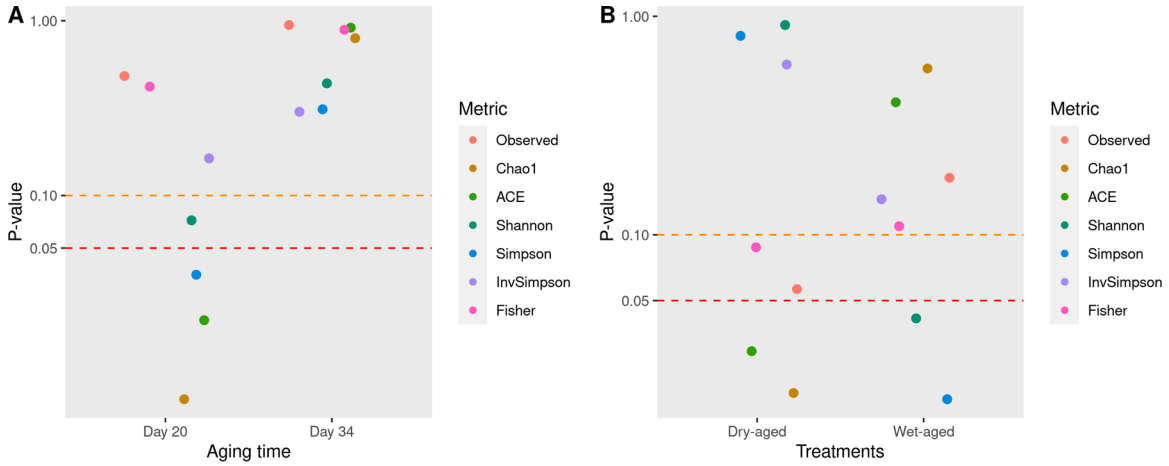

Fig. 2. Scatter plots of p-values of the models evaluating the effects of aging treatment and time points on meat alpha-diversity indices (corrected for baseline). The dashed line in red represents the p-value threshold equal to 0.05, and in orange, equivalent to 0.10. A. distribution of p-values from the linear model (1), the effect of treatment per time point; B. distribution of p-values from the linear model (2), the effect of time point within the treatment.

3.3.2. Beta diversity

Sample correlations were assessed using the weighted Unifrac distances derived from the beta diversity analysis. Fig. 3 illustrates the distribution of samples across the initial two dimensions of the multi-dimensional scaling based on unweighted Unifrac distances. The results revealed distinct clusters between the two aging processes; however, there was no evident clustering when examining the aging periods, but no apparent clustering when evaluating the aging periods.

4. Discussion

Traditional microbiological analyses showed an influence of aging time and type on fresh meat (Table 1). The increase ($P < 0.05$) in counts of mesophilic bacteria, psychrotrophic bacteria, and lactic acid bacteria in wet-aged meat compared to fresh meat can mainly be justified by the high-water activity of the meat and the microaerophilic conditions resulting from storing the meat in anaerobic packaging with low oxygen pressure; which are ideal conditions for the multiplication and growth of these microbial groups (Hwang et al., 2022).

The significant declines observed in counts of mesophilic bacteria and psychrotrophic bacteria, as well as the 1-log reduction in lactic acid bacteria, in dry-aged samples aged for 34 days, compared to fresh meat and wet-aged samples, are related to the decrease in surface water activity. This is due to the absence of packaging during aging, which leads to dehydration of the meat's surface, reducing the water activity; which alters the ideal growth conditions for various microorganisms (Hwang et al., 2022; Da Silva Bernardo et al., 2021). The decrease in water activity also explains the reduction of 1-log cycle in mesophilic bacteria when dry-aged samples were aged for more than 20 days.

No significant effect was observed among the samples for enterobacteria, molds, and yeasts, with variations of less than 1-log cycle between the treatments for both microbial groups.

Interestingly, the microbial species identified by sequencing agree with traditional microbiological results, as the identified species are representative of mesophilic, psychotropic, and LAB. *Romboutsia* (2.8 %) (Ricaboni et al., 2016), *Candidatus Nitrosotalea* (2.4 %) (Prosser and Nicol, 2016), *Akkermansia* (2.3 %) (Derrien et al., 2004), *Lactobacillus* (5.4 %) (Hammes and Hertel, 2015), and *Leuconostoc* (2.5 %) (Holzapfel et al., 2015) are known for their mesophilic growth; *Lactobacillus* and *Leuconostoc* produce lactic acid as an end product from glucose, as does *Carnobacterium* (47.9 %) (psychrotolerant, with growth temperature of 0 °C, 30 °C, and 40 °C) and are therefore also LAB species (Hammes and Hertel, 2015; Holzapfel et al., 2015; Hammes and Hertel, 2015). Lee et al. (2018) also observed a significant increase in lactic acid bacteria counts in dry-aged samples during the first few days of maturation ($p < 0.05$). LAB is also known to influence the flavor of aged meat (Lee et al., 2018).

Pseudomonades are a species that is both mesophilic (growth at 28 °C) and psychrotrophic (optimal growth at 4 °C) (Palleroni, 2015), which leads us to speculate that in this work, *Pseudomonas* may be representing the psychrotrophic group. (Lee et al., 2019) also observed many psychrotrophic bacteria at the beginning of the dry-aging process, which increased afterward. Overall, we noted a higher prevalence of *Pseudomonas* (22.2 %) in the metagenomics analysis for both dry-aging periods and in the traditional microbiological analysis after 20 days of aging, represented as Psychrotrophic (Table 1). *Pseudomonas* spp. is one of the main microorganisms responsible for meat spoilage under aerobic conditions due to its high affinity for oxygen and ability to multiply at low temperatures (McSharry et al., 2021). This group is commonly found in environments such as water, soil, vegetables, raw milk, and frozen meats, and can grow at a wide range of temperatures (from 1.5 to 42 °C), which favors their growth on various food types (Palleroni, 2015). (Capouya et al., 2020) observed a predominance of *Pseudomonas* in dry-aged samples, corroborating the bioinformatics results. Other works have already demonstrated the presence of *Pseudomonas* in dry-aged samples (Gowda et al., 2022; Kim et al., 2021).

The wet-aged samples showed a higher total bacterial count at the end of the process, which can be explained by the higher water activity, favoring the exponential development of microorganisms (Syamaladevi et al., 2016). Moreover, packaging may favor the development of *Carnobacterium* (47.9 %), a genus of the order Lactobacillales, commonly found in frozen and vacuum-packed meat products (Odeyemi et al., 2020). Its low affinity for O_2 shows that the barrier packaging did indeed prevent contact with the external environment (Zhang et al., 2018), which also favored the development of other anaerobic LABs, such as *Leuconostoc*. According to (Zhang et al., 2018), three main factors, in isolation or associated, could explain the prevalence of *Carnobacterium* after longer periods: 1) its slower growth rate, 2) lesser extent growth, and 3) antimicrobial potential, as other studies demonstrated the ability of some *Carnobacterium* species to produce antibacterial compounds (dos Reis et al., 2011; Hammi et al., 2016).

Another interesting finding is the identification of *Carnobacterium*. Their survival is linked to their ability to metabolize arginine and other carbohydrates (Leisner et al., 2007). Moreover, some *Carnobacterium* species can produce antibacterial compounds that inhibit the growth of other spoilage or pathogenic bacteria in food (Zhang et al., 2018; Castellano et al., 2008). When analyzing the metagenomics results, a high abundance of the genus *Carnobacterium* in the wet-aged samples is shown. This LAB group grows best under anaerobic conditions and at low temperatures (Leisner et al., 2007; Lo and Sheth, 2021), the characteristic environment for wet-aging; a result corroborated by microbiological testing. Their ability to also produce acid changes the pH of the

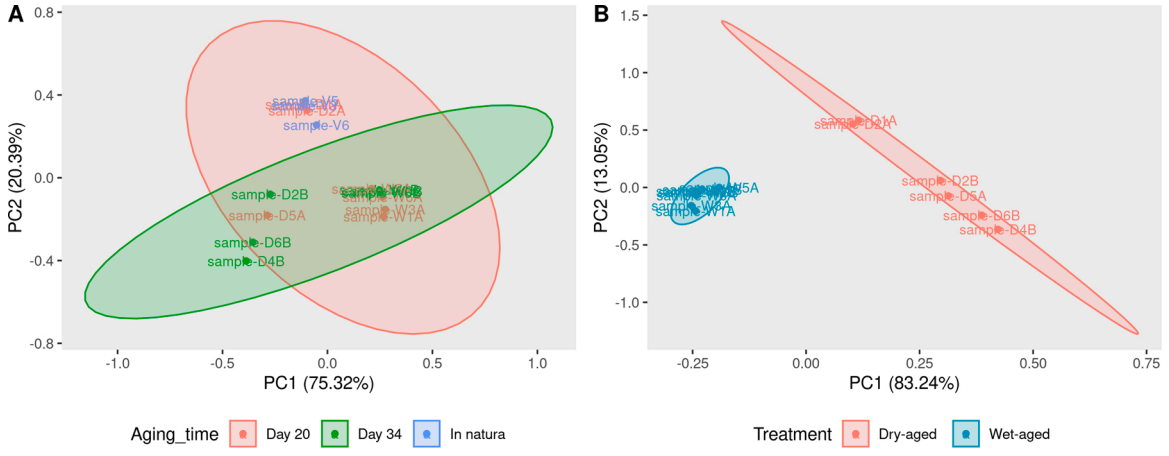

Fig. 3. A. Beta diversity cluster comparing the samples before, during, and after the aging process. **B.** Beta diversity cluster compares the *in natura*, dry-, and wet-aged samples.

environment and controls the growth of other bacteria, which may have favored their prevalence (Ribeiro et al., 2021).

(Capouya et al., 2020) also observed a higher count of *Pseudomonas* OTUs in dry-aged samples than in wet-aged samples, corroborating the present study. Moreover, we noted a significant variation in the Shannon index when comparing the processes. Aside from external factors, such as temperature, light, and humidity, which can affect the microbiome of aged meat (Doster et al., 2020), the location of sample collection can influence the bacterial diversity of each sample (Capouya et al., 2020).

The *Candidatus* genus, found in the *in natura* and dry-aged (20 days) samples, represents a group of ammonia-oxidizing archaea. These nitrifiers oxidize ammonia (NH_3) into nitrite (NO_2) (v. Zhalnina et al., 2014) and perform critical environmental roles in open oceans, soils, arctic, hot springs, and marine sponges. This microorganism is usually present in the soil, but studies have indicated its presence in meat, which may have been contaminated by the environment. Although easily identifiable by molecular testing, the genus does not appear in traditional microbiological tests because they are not easily cultured (Guindo et al., 2020; Muriuki et al., 2021).

The different aging methods considerably change the meat microbial composition, as shown by beta diversity analysis. Within-sample microbial population diversity varies more strikingly between time points (3 indices – ACE, Chao1, and Simpson had a p-value less than 0.05) than between treatments, as demonstrated by the similarly distributed relevance (2 indices each with significance less than 0.05). These findings are corroborated by (Capouya et al., 2020), who found that time was a more relevant influence on the results than aging treatments, sample position, or category variables.

Despite the better flavor provided by dry-aging, this process is still more costly than wet-aging and might make this product inaccessible to the final consumer.

5. Conclusion

Our findings demonstrate that both traditional microbiological analysis and NGS effectively identify and quantify microorganisms (mesophilic, psychrotrophic, LAB, molds, and yeasts) in dry- and wet-aged beef, allowing for comparisons with fresh samples. However, given the significant impact of microbiota on meat quality and safety, and considering the varying effects of aging methods on microbiota, NGS emerges as the superior evaluation method, providing more specific results. Notably, NGS technology revealed detailed insights into specific species like *Akkermansia, Carnobacterium, Candidatus Nitrosotalea, Lactobacillus, Leuconostoc, Pseudomonas*, and *Romboutsia*; known for causing food spoilage and resulting in economic losses.

Furthermore, we investigated microbial variations within both aging processes, utilizing both traditional tests and NGS to discern the presence of pathogens and spoilage groups in food. In summary, both traditional microbiology testing and NGS are crucial techniques for identifying microorganism levels in aged meat. Despite their data, NGS was demonstrated to be more precise.

CRediT authorship contribution statement

Anderson Clayton da Silva Abreu: Data curation, Investigation, Methodology, Validation, Writing – review & editing. **Luiz Gustavo de Matos:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft. **Nathália Cristina Cirone Silva:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. **Marcelo Mendes Brandão:** Software, Writing – review & editing. **Natália Faraj Murad:** Software, Writing – review & editing. **Chiara Gini:** Software, Validation, Writing – review & editing. **Jonatã Henrique Rezende de Souza:** Software, Validation, Writing – review & editing. **Sérgio Bertelli Pflanzer Jr.:** Data curation, Investigation, Methodology, Writing – review & editing. **Maristela da Silva do Nascimento:** Data curation, Writing – review & editing. **Juliano Leonel Gonçalves:** Software, Writing – original draft. **Vanessa Pereira Perez Alonso:** Software, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This study was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES, Brasília, Brazil; grant numbers 001). The authors thank Espaço da Escrita – Pró-Reitoria de Pesquisa – UNICAMP (Campinas, Brazil) for the language services provided.

References

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32–46. https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x.

APHA, 2001. Compendium Methods Microbiological Examination Foods.

Barcenilla, C., Ducic, M., López, M., Prieto, M., Álvarez-Ordóñez, A., 2022. Application of lactic acid bacteria for the biopreservation of meat products: a systematic review. Meat Sci. 183 https://doi.org/10.1016/j.meatsci.2021.108661.

Biscarini, F., Palazzo, F., Castellani, F., Masetti, G., Grotta, L., Cichelli, A., Martino, G., 2018. Rumen microbiome in dairy calves fed copper and grape-pomace dietary supplementations: Composition and predicted functional profile. PLoS One 13, e0205670. https://doi.org/10.1371/journal.pone.0205670.

Capouya, R., Mitchell, T., Clark, D.I., Clark, D.L., Bass, P., 2020. A survey of microbial communities on dry-aged beef in commercial meat processing facilities. Meat Muscle Biol. 4 https://doi.org/10.22175/mmb.10373.

Castellano, P., Belfiore, C., Fadda, S., Vignolo, G., 2008. A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. Meat Sci. 79, 483–499. https://doi.org/10.1016/j.meatsci.2007.10.009.

A. Chao, 1984. Nonparametric Estimation of the Number of Classes in a Population.

Chao, A., Lee, S.-M., 1989. Estimating the number of classes via sample coverage. Theory Methods 210–217.

Chao, A., Yang, M.C.K., 1993. Stopping rules and estimation for recapture debugging with unequal failure rates. Biometrika 80, 193–201. https://doi.org/10.1093/biomet/80.1.193.

da Silva Abreu, A.C., Matos, L.G., da Silva Cândido, T.J., Barboza, G.R., de Souza, V.V.M. A., Munive Nuñez, K.V., Cirone Silva, N.C., 2021. Antimicrobial resistance of Staphylococcus spp. isolated from organic and conventional Minas Frescal cheese producers in São Paulo, Brazil. J. Dairy Sci. 104, 4012–4022. https://doi.org/10.3168/jds.2020-19338.

Da Silva Bernardo, A.P., Marinelli Saraiva Ferreira, F., Muniz Da Silva, A.C., Souza Prestes, F., Francisco, V.C., Tieko Nassu, R., Da Silva Do Nascimento, M., Bertelli Pflanzer, S., 2021. Dry-aged and wet-aged beef: effects of aging time and temperature on microbiological profile, physicochemical characteristics, volatile compound profile and weight loss of meat from Nellore cattle (Bos indicus). Anim. Prod. Sci. 61, 1497–1509. https://doi.org/10.1071/AN20120.

Dashdorj, D., Tripathi, V.K., Cho, S., Kim, Y., Hwang, I., 2016. Dry aging of beef; review. J. Anim. Sci. Technol. 58 https://doi.org/10.1186/s40781-016-0101-9.

Derrien, M., Vaughan, E.E., Plugge, C.M., de Vos, W.M., 2004. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int. J. Syst. Evol. Microbiol 54, 1469–1476. https://doi.org/10.1099/ijs.0.02873-0.

Di Paolo, M., Ambrosio, R.L., Lambiase, C., Vuoso, V., Salzano, A., Bifulco, G., Barone, C. M.A., Marrone, R., 2023. Effects of the aging period and method on the physicochemical, microbiological and rheological characteristics of two cuts of charolais beef. Foods 12, 531. https://doi.org/10.3390/foods12030531.

dos Reis, F.B., de Souza, V.M., Thomaz, M.R.S., Fernandes, L.P., de Oliveira, W.P., de Martinis, E.C.P., 2011. Use of Carnobacterium maltaromaticum cultures and hydroalcoholic extract of Lippia sidoides Cham. against Listeria monocytogenes in fish model systems. Int J. Food Microbiol 146, 228–234. https://doi.org/10.1016/j.ijfoodmicro.2011.02.012.

Doster, E., Thomas, K.M., Weinroth, M.D., Parker, J.K., Crone, K.K., Arthur, T.M., Schmidt, J.W., Wheeler, T.L., Belk, K.E., Morley, P.S., 2020. Metagenomic Characterization of the Microbiome and Resistome of Retail Ground Beef Products. Front Microbiol 11. https://doi.org/10.3389/fmicb.2020.541972.

R.A. Fisher, A.S. Corbet, C.B. Williams, 1943. The Relation Between the Number of Species and the Number of Individuals in a Random Sample of an Animal Population. https://www.jstor.org/stable/1411.

U.S. Food and Drugs Administration, Bacteriological Analytical Manual, 8th ed., 1998.

Glöckner, F.O., Yilmaz, P., Quast, C., Gerken, J., Beccati, A., Ciuprina, A., Bruns, G., Yarza, P., Peplies, J., Westram, R., Ludwig, W., 2017. 25 years of serving the community with ribosomal RNA gene reference databases and tools. J. Biotechnol. 261, 169–176. https://doi.org/10.1016/j.jbiotec.2017.06.1198.

Gowda, T.K.G.M., De Zutter, L., Van Royen, G., Van Damme, I., 2022. Exploring the microbiological quality and safety of dry-aged beef: A cross-sectional study of loin surfaces during ripening and dry-aged beef steaks from commercial meat companies in Belgium. Food Microbiol 102, 103919. https://doi.org/10.1016/J.FM.2021.103919.

Guindo, C.O., Davoust, B., Drancourt, M., Grine, G., 2020. Diversity of methanogens in animals' gut. Microorganisms 9, 13. https://doi.org/10.3390/microorganisms9010013.

Hammes, W.P., Hertel, C., 2015. Lactobacillus. In: Bergey's Manual of Systematics of Archaea and Bacteria. Wiley, pp. 1–76. https://doi.org/10.1002/9781118960608.gbm00604.

Hammes, W.P., Hertel, C., 2015. Carnobacterium. In: Bergey's Manual of Systematics of Archaea and Bacteria. Wiley, pp. 1–14. https://doi.org/10.1002/9781118960608.gbm00593.

Hammi, I., Delalande, F., Belkhou, R., Marchioni, E., Cianferani, S., Ennahar, S., 2016. Maltaricin CPN, a new class IIa bacteriocin produced by *Carnobacterium maltaromaticum* CPN isolated from mould-ripened cheese. J. Appl. Microbiol. 121, 1268–1274. https://doi.org/10.1111/jam.13248.

Holzapfel, WilhelmH., Björkroth, J.A., Dicks, L.M.T., 2015. Leuconostoc. In: Bergey's Manual of Systematics of Archaea and Bacteria. Wiley,, pp. 1–23. https://doi.org/10.1002/9781118960608.gbm00607.

Hwang, S.H., Lee, J., Nam, T.G., Koo, M., Cho, Y.S., 2022. Changes in physicochemical properties and bacterial communities in aged Korean native cattle beef during cold storage. Food Sci. Nutr. 10, 2590–2600. https://doi.org/10.1002/fsn3.2864.

Kim, S., Kim, J.C., Park, S., Kim, J., Yoon, Y., Lee, H., 2021. Identification of microbial flora in dry aged beef to evaluate the rancidity during dry aging. Processes 9, 2049. https://doi.org/10.3390/PR9112049/S1.

Lee, H.J., Yoon, J.W., Kim, M., Oh, H., Yoon, Y., Jo, C., 2019. Changes in microbial composition on the crust by different air flow velocities and their effect on sensory properties of dry-aged beef. Meat Sci. 153, 152–158. https://doi.org/10.1016/j.meatsci.2019.03.019.

H.J. Lee, J. Choe, J.W. Yoon, S. Kim, H. Oh, Y. Yoon, C. Jo, Determination of Salable Shelf-life for Wrappackaged Dry-aged Beef during Cold Storag, Korean J Food Sci Anim Resour. (2018).

Leisner, J.J., Laursen, B.G., Prévost, H., Drider, D., Dalgaard, P., 2007. *Carnobacterium:* positive and negative effects in the environment and in foods. FEMS Microbiol Rev. 31, 592–613. https://doi.org/10.1111/j.1574-6976.2007.00080.x.

Lo, C.K.-L., Sheth, P.M., 2021. Carnobacterium inhibens isolated in blood culture of an immunocompromised, metastatic cancer patient: a case report and literature review. BMC Infect. Dis. 21, 403 https://doi.org/10.1186/s12879-021-06095-7.

Lopez-Caballero, M., Sanchez-Fernandez, J., Moraí, A., 2001. Growth and metabolic activity of Shewanella putrefaciens maintained under different CO and O concentrations 2 2 †. www. elsevier. nl/locate/ijfoodmicro.

López-Pedrouso, M., Rodríguez-Vázquez, R., Purriños, L., Oliván, M., García-Torres, S., Sentandreu, M.Á., Lorenzo, J.M., Zapata, C., Franco, D., 2020. Sensory and physicochemical analysis of meat from bovine breeds in different livestock production systems, pre-slaughter handling conditions, and ageing time. Foods 9. https://doi.org/10.3390/foods9020176.

Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J., Knight, R., 2011. UniFrac: an effective distance metric for microbial community comparison. ISME J. 5, 169–172. https://doi.org/10.1038/ismej.2010.133.

McSharry, S., Koolman, L., Whyte, P., Bolton, D., 2021. The microbiology of beef from carcass chilling through primal storage to retail steaks. Curr. Res. Food Sci. 4, 150–162. https://doi.org/10.1016/j.crfs.2021.03.002.

Muriuki, S.W., Rengan, M.S., Budambula, N.L.M., 2021. Prokaryotic diversity and potentially pathogenic bacteria in vended foods and environmental samples. Ann. Microbiol. 71, 27 https://doi.org/10.1186/s13213-021-01640-w.

Odeyemi, O.A., Alegbeleye, O.O., Strateva, M., Stratev, D., 2020. Understanding spoilage microbial community and spoilage mechanisms in foods of animal origin. Compr. Rev. Food Sci. Food Saf. 19, 311–331. https://doi.org/10.1111/1541-4337.12526.

Oh, H., Lee, H.J., Lee, J., Jo, C., Yoon, Y., 2019. Identification of microorganisms associated with the quality improvement of dry-aged beef through microbiome analysis and DNA sequencing, and evaluation of their effects on beef quality. J. Food Sci. 84, 2944–2954. https://doi.org/10.1111/1750-3841.14813.

Palleroni, N.J., 2015. Pseudomonas. In: Bergey's Manual of Systematics of Archaea and Bacteria. Wiley,. https://doi.org/10.1002/9781118960608.gbm01210.

Palleroni, N.J., 2015. Burkholderia. In: Bergey's Manual of Systematics of Archaea and Bacteria. Wiley,, pp. 1–50. https://doi.org/10.1002/9781118960608.gbm00935.

Paulson, J.N., Stine, O.C., Bravo, H.C., Pop, M., 2013. Differential abundance analysis for microbial marker-gene surveys. Nat. Methods 10, 1200–1202. https://doi.org/10.1038/nmeth.2658.

Prosser, J.I., Nicol, G.W., 2016. *Candidatus* Nitrosotalea. In: Bergey's Manual of Systematics of Archaea and Bacteria. Wiley,, pp. 1–7. https://doi.org/10.1002/9781118960608.gbm01292.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41, D590–D596. https://doi.org/10.1093/nar/gks1219.

R.K. Ravi, K. Walton, M. Khosroheidari, MiSeq: A Next Generation Sequencing Platform for Genomic Analysis, in: 2018: pp. 223–232. https://doi.org/10.1007/978-1-4939-7471-9_12.

Ribeiro, F.A., Lau, S.K., Furbeck, R.A., Herrera, N.J., Henriott, M.L., Bland, N.A., Fernando, S.C., Subbiah, J., Sullivan, G.A., Calkins, C.R., 2021. Ultimate pH effects on dry-aged beef quality. Meat Sci. 172, 108365 https://doi.org/10.1016/j.meatsci.2020.108365.

Ricaboni, D., Mailhe, M., Khelaifia, S., Raoult, D., Million, M., 2016. Romboutsia timonensis, a new species isolated from human gut. N. Microbes N. Infect. 12, 6–7. https://doi.org/10.1016/j.nmni.2016.04.001.

RStudio Team, RStudio: Integrated Development for R. RStudio, (2020). http://www.rstudio.com/. (Accessed 11 December 2022).

Ryu, S., Park, M.R., Maburutse, B.E., Lee, W.J., Park, D.-J., Cho, S., Hwang, I., Oh, S., Kim, Y., 2018. Diversity and characteristics of the meat microbiological community on dry aged beef. J. Microbiol Biotechnol. 28, 105–108. https://doi.org/10.4014/jmb.1708.08065.

Salzano, A., Cotticelli, A., Marrone, R., D'Occhio, M.J., D'Onofrio, N., Neglia, G., Ambrosio, R.L., Balestrieri, M.L., Campanile, G., 2021. Effect of breeding techniques and prolonged post dry aging maturation process on biomolecule levels in raw buffalo meat. Vet. Sci. 8, 66. https://doi.org/10.3390/vetsci8040066.

Shannon, C.E., 1948. A mathematical theory of communication. Bell Syst. Tech. J. 27, 379–423. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x.

Shi, Y., Zhang, W., Zhou, G., 2020. Effects of different moisture-permeable packaging on the quality of aging beef compared with wet aging and dry aging. Foods 9. https://doi.org/10.3390/foods9050649.

Simpson, E.H., 1949. Measurement of diversity. Nature 163. https://doi.org/10.1038/163688a0, 688–688.

B. Smith, J.B. Wilson, A Consumer's Guide to Evenness Indices, 1996. https://about.jstor.org/terms.

Syamaladevi, R.M., Tang, J., Villa-Rojas, R., Sablani, S., Carter, B., Campbell, G., 2016. Influence of water activity on thermal resistance of microorganisms in low-moisture foods: a review. Compr. Rev. Food Sci. Food Saf. 15, 353–370. https://doi.org/10.1111/1541-4337.12190.

Terjung, N., Witte, F., Heinz, V., 2021. The dry aged beef paradox: why dry aging is sometimes not better than wet aging. Meat Sci. 172 https://doi.org/10.1016/j.meatsci.2020.108355.

Tyuftin, A.A., Kerry, J.P., 2023. The storage and preservation of meat: storage and packaging. In: Lawrie's Meat Science. Elsevier, pp. 315–362. https://doi.org/10.1016/B978-0-323-85408-5.00017-0.

v. Zhalnina, K., Dias, R., Leonard, M.T., Dorr de Quadros, P., Camargo, F.A.O., Drew, J. C., Farmerie, W.G., Daroub, S.H., Triplett, E.W., 2014. Genome sequence of candidatus nitrososphaera evergladensis from Group I.1b enriched from everglades soil reveals novel genomic features of the ammonia-oxidizing archaea. PLoS One 9, e101648. https://doi.org/10.1371/journal.pone.0101648.

Valoppi, F., Agustin, M., Abik, F., Morais de Carvalho, D., Sithole, J., Bhattarai, M., Varis, J.J., Arzami, A.N.A.B., Pulkkinen, E., Mikkonen, K.S., 2021. Insight on current advances in food science and technology for feeding the world population. Front Sustain Food Syst. 5 https://doi.org/10.3389/fsufs.2021.626227.

van Reckem, E., de Vuyst, L., Weckx, S., Leroy, F., 2021. Next-generation sequencing to enhance the taxonomic resolution of the microbiological analysis of meat and meat-derived products. Curr. Opin. Food Sci. 37, 58–65. https://doi.org/10.1016/j.cofs.2020.09.004.

Xu, M.M., Kaur, M., Pillidge, C.J., Torley, P.J., 2022. Microbial biopreservatives for controlling the spoilage of beef and lamb meat: their application and effects on meat quality. Crit. Rev. Food Sci. Nutr. 62, 4571–4592. https://doi.org/10.1080/10408398.2021.1877108.

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glöckner, F.O., 2014. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucleic Acids Res. 42, D643–D648. https://doi.org/10.1093/nar/gkt1209.

You, W., Henneberg, R., Saniotis, A., Ge, Y., Henneberg, M., 2022. Total meat intake is associated with life expectancy: a cross-sectional data analysis of 175 contemporary populations. Int. J. Gen. Med. 15, 1833–1851. https://doi.org/10.2147/IJGM.S333004.

Zagorec, M., Champomier-Vergès, M.-C., 2023. Meat microbiology and spoilage. In: Lawrie's Meat Science. Elsevier, pp. 195–218. https://doi.org/10.1016/B978-0-323-85408-5.00011-X.

Zhang, P., Badoni, M., Gänzle, M., Yang, X., 2018. Growth of Carnobacterium spp. isolated from chilled vacuum-packaged meat under relevant acidic conditions. Int. J. Food Microbiol. 286, 120–127. https://doi.org/10.1016/j.ijfoodmicro.2018.07.032.