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Microbiome mapping in beef processing reveals safety-relevant variations in microbial diversity and genomic features

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ABSTRACT

The microbiome of surfaces along the beef processing chain represents a critical nexus where microbial ecosystems play a pivotal role in meat quality and safety of end products. This study offers a comprehensive analysis of the microbiome along beef processing using whole metagenomics with a particular focus on antimicrobial resistance and virulence-associated genes distribution. Our findings highlighted that microbial communities change dynamically in the different steps along beef processing chain, influenced by the specific conditions of each micro-environment. *Brochothrix thermosphacta*, *Carnobacterium maltaromaticum*, *Pseudomonas fragi*, *Psychrobacter cryohalolentis* and *Psychrobacter immobilis* were identified as the key species that characterize beef processing environments. Carcass samples and slaughterhouse surfaces exhibited a high abundance of antibiotic resistance genes (ARGs), mainly belonging to aminoglycosides, β-lactams, amphenicols, sulfonamides and tetracyclines antibiotic classes, also localized on mobile elements, suggesting the possibility to be transmitted to human pathogens.

We also evaluated how the initial microbial contamination of raw beef changes in response to storage conditions, showing different species prevailing according to the type of packaging employed. We identified several genes leading to the production of spoilage-associated compounds, and highlighted the different genomic potential selected by the storage conditions.

Our results suggested that surfaces in beef processing environments represent a hotspot for beef contamination and evidenced that mapping the resident microbiome in these environments may help in reducing meat microbial contamination, increasing shelf-life, and finally contributing to food waste restraint.

1. Introduction

Microbial contamination in food processing environments is a critical factor that strongly affects food quality and safety (De Filippis et al., 2021). Specific microbial consortia are associated with food processing environments and must be balanced in order to obtain safe products with high quality standards (Sabater et al., 2021). Indeed, food processing environmental microbiota may be easily transferred to the final product and actively participate in defining the safety and quality of the products (Ferrocino et al., 2022). The beef production chain is considered particularly hazardous for the spreading of microbial contamination. Each phase, including the slaughtering of the animals, the processing and portioning of the carcasses, their maturation and handling in the retail shop are all steps where a potential cross-contamination with the environmental microbiota may occur (Sequino et al., 2022). Meat represents a valuable source of high biological value proteins, iron, vitamins and minerals. However, its high-water activity and composition rich in nutrients make it a perfect environment for the growth of a wide range of microorganisms, both spoilers and pathogens (Pellissery et al., 2020). Fresh meat microbiota is highly complex

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(Sequino et al., 2022), but not all the members of the initial community will contribute to spoilage, only a fraction of this initial community will be able to dominate in the specific micro-ecosystem generated after meat processing. These microbes recognized as Specific Spoilage Organisms (SSOs) will be selected by intrinsic and extrinsic ecological factors, such as pH, O_2 availability, temperature and interactions with other members of the microbiota (Nychas et al., 2008). Among the most common meat spoilers, *Pseudomonas* spp., *Brochothrix thermosphacta,* species of the *Enterobacteriaceae* family and lactic acid bacteria (the former *Lactobacillus*, *Carnobacterium* and *Leuconostoc* spp.) are recognized as the principal players in meat decay (Raimondi et al., 2018). During meat spoilage, they may produce a wide range of metabolites (aldehydes, ketones, esters, alcohols, organic acids, amines and sulfur compounds) that, above a certain threshold, make the meat unacceptable for human consumption (Casaburi et al., 2015). The use of low temperature during storage, coupled with modified-atmosphere or vacuum packaging can affect microbial associations and dynamics, impacting on the spoilage rate (Ercolini et al., 2011; Doulgeraki et al., 2012; Potakos *et al.*, 2015).

The environmental microbiota from meat processing plants has frequently been discussed as a primary source of microorganisms that can affect, quality and safety of meat (Hultman et al., 2015; De Filippis et al., 2013; Stellato et al., 2016; Zwirzitz et al., 2020). Indeed, the presence of organic residues on surfaces can promote microbial proliferation and aggregation (Giaouris et al., 2014). The most abundant species present on processing tools are often found at high levels on meat, suggesting the establishment of an equilibrium between food and the environment that affects the quality of the final product (Vihavainen et al., 2007; De Filippis et al., 2013). In addition, several microorganisms, including *Pseudomonas* spp. (Cunault et al., 2018), are able to produce biofilms on food industry surfaces, where other microbial cells remain embedded and are protected from cleaning procedures (Nikolaev et al., 2022). The presence of biofilms has been shown to enhance microbial antibiotic resistance (AR) and to promote the transmission of AR genes (ARGs) (Bowler et al., 2020; Abebe, 2020). Indeed, the spreading of ARGs along the food chains is a major concern for public health (Likotrafiki et al., 2018; Sagar et al., 2023; Yuan et al., 2023). While several studies reported the isolation of AR bacteria from food industry surfaces, only few of them applied a metagenomics-based screening of the whole resistome (Valentino et al., 2022; Cobo-Diaz *et al.*, 2021). In particular, it was recently highlighted that pig slaughterhouse surfaces represent an important reservoir of ARGs, that can be easily transferred to the meat microbiota (Cobo-Diaz *et al.*, 2021). The spreading of AR in the meat chain may be linked with the overuse of antimicrobials and antibiotics administered at the farm level (Van Boeckel et al., 2015) and beef cattle microbiome is a hotspot for AR transmission (Wang et al., 2023; Auffret et al., 2017). Indeed, during slaughtering and carcass portioning, ARG-carrying bacteria may contaminate directly the meat, or tools and surfaces, where they may become resident. Therefore, identifying routes of contamination and AR spread along the fresh beef chain may help to design appropriate cleaning procedures and plans to monitor and control the resistome evolution.

The purpose of this work is to assess, through whole metagenomics, the taxonomical distribution patterns, the antimicrobial resistance and the virulence potential of the resident microbiome along a beef processing chain, from the abattoir to the retail. In addition, the effects of different packaging and storage conditions on microbiome dynamics during meat shelf-life were also evaluated.

2. Materials and methods

2.1. Samples collection and packaging preparation

One beef processing facility located in the county of Xanthi (Greece) was visited in May 2021 after the completion of the routinary cleaning procedures and food contact (FC) and non-food contact (NFC) surfaces were sampled using Whirl-Pak Hydrated PolyProbe swabs (Whirl-Pak, Madison, Wisconsin, US), covering an area of about 1 m^2, or a sampling unit (e.g., one knife, one table). In particular, swabs were collected from carcasses just after the slaughtering, from hands of the slaughterhouse and the retail operators and from slaughterhouse and retail butcher shop surfaces.

A total of 56 samples were taken, including carcass swabs at the beginning of the processing (n = 9), environmental FC swabs (n = 36), NFC swabs (n = 6) and swabs from hands/aprons of employees (n = 5). All the samples were stored at −80 °C and shipped in dry ice to the University of Naples Federico II (Italy), where they were processed.

In an additional experiment, in order to evaluate the effect of different packaging and storage conditions in microbial dynamics during storage, fresh raw beef was bought in a butcher shop located in Campania region (Southern Italy). Two different samplings were carried out in October and December 2022. A beef cut corresponding to "brisket" was cut in chops of about 40 g in the butcher shop, transported to the laboratory within 30 min and then packed in trays (Coopbox, Bologna, Italy, 500 cc) with an absorbent sheet to avoid accumulation of exudates.

Samples from the same beef cut were packed aerobically (AIR), in modified atmosphere packaging (MAP; gas mixture 60 kPa O_2, 30 kPa CO_2 and 10 kPa N_2) and vacuum packaging (VP). AIR and MAP samples were packed by using packaging machine (TSM 105 Minipack-Torre, Dalmine, Bergamo, Italy). The film used was a multilayer film of PA/EVOH/PE ($PO_2 = 1.3\ cm^3\ m^{-2}\ 24\ h^{-1}\ atm^{-1}$ at 23 °C, 0 % RH; thickness: 54 μm) and the ratio between the volume of gas and weight of food product (G/P ratio) was 4:1 (V/W). VP samples were placed in the tray and vacuum packed using bags of plastic barrier film (200 x 300 mm) co-extruded, copolymer of vinylidene chloride (VDC) and Ethyl Vinyl Alcohol (EVA) as barrier layer, and low density polyethylene (LDPE, CRYOVAC BB3050, oxygen transmission 0.83 $cm^3\ m^{-2}h^{-1}$ at 23 °C, provided by CRYOVAC Sealed Air S.r.l., Milano, Italy) and vacuum packaging machine (Lavezzini Model Jolly new gas, Fiorenzuola D'Arda, Piacenza, Italy).

All samples (n = 70) were stored at three different temperatures (0, 4, 10 °C) and analysed at different time points. Samples at 0 and 4 °C were stored up to 10 and 7 days (aerobically), respectively and 16 days (MAP and VP). Samples at 10 °C were stored up to 7 days in all packaging conditions, since the deterioration was evident after this period.

For air and modified atmosphere packaging conditions, the headspace gas composition was monitored using a O_2/CO_2 gas analyzer (accuracy of 0.5 %), equipped with a needle (Check Mate 9900 O_2/CO_2; Ringsted, Denmark) during storage at 0 and 10 °C (Table S1).

2.2. DNA extraction and whole metagenome sequencing

Swabs' samples were pre-processed by adding 10 ml of STE (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) buffer to each sample. Microbial cells were detached from swab surfaces using a Stomacher (300 rpm × 30 s), then the supernatant was collected and aliquoted in 5 mL sterile tubes (Eppendorf, Hamburg, Germany). In addition, fresh beef samples were weighted (~30g) and transferred to a sterile bag, where STE buffer was added in 5:1 ratio and microorganisms were detached from the surface of the meat by shaking, without damaging the tissues and limiting the release of meat epithelial cells. About 100 mL of STE solution containing the microorganisms were collected. The tubes were centrifuged at 12.000 × g for 2 min, then the cellular pellet was washed twice with 2 mL of sterile STE and stored at −80 °C until DNA extraction. DNA extraction was performed from the pellets using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and quantified using the Qubit HS Assay (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

Metagenomic libraries were prepared using the Nextera XT Index Kit v2 (Illumina, San Diego, California, United States), then whole

metagenome sequencing was performed on an Illumina NovaSeq platform, leading to 2 × 150 bp, paired-end reads.

2.3. Bioinformatic analysis

Host reads contamination was removed mapping reads to the Bos Taurus genome (NCBI Accession Number: PRJNA391427) by using the Best Match Tagger (BMTagger; https://www.hmpdacc.org/hmp/doc/HumanSequenceRemoval_SOP.pdf). The resulting reads were quality-checked and filtered through Prinseq-lite v. 0.20.4, using parameters '-trim_qual_right 5′ and '-min_len 60′ (Schmieder & Edwards, 2011). Taxonomic and functional profiles were obtained through MetaPhlAn v. 4.0 (Blanco-Míguez et al., 2023) and HUMAnN v. 3.6 (Beghini et al., 2021), respectively. Genes and pathways from HUMAnN outputs were relabeled according to the UniRef90 annotations.

For each sample, high-quality reads were assembled into contigs using MEGAHIT v. 1.2.2 (Li et al., 2015), filtering out contigs < 1,000 bp. Reads from each sample were mapped to the corresponding contigs using Bowtie2 v. 2.2.9 (Langmead & Salzberg, 2012), with parameters '–very-sensitive-local' and '–no-unal'. MetaBAT v. 2.12.1 (Kang et al., 2019) was used for binning contigs > 1,500 bp into Metagenome-Assembled Genomes (MAGs). The CheckM "lineage_wf" workflow v. 1.1.3 (Parks et al., 2015), was used to assess the quality of MAGs and only those with ≥ 50 % completeness and < 5 % contamination (Pasolli et al., 2019) were retained for further analyses.

Pairwise Mash distances (v. 2.0; option "-s 10000" for sketching; Ondov et al., 2016) were computed between the MAGs and a 5 % dissimilarity threshold was used to assign MAGs to a Species-level Genome Bin (SGB), as previously suggested (Pasolli et al., 2019). Taxonomy was inferred by comparing the most complete and less contaminated MAG from each SGB to the MetaRefSGB database (December 2020 release; Pasolli et al., 2019), selecting 5 %, 15 % and 30 % dissimilarity threshold for species, genus and family level, respectively.

Furthermore, reads and metagenome assemblies coming from the beef processing facility were screened for AR and Virulence Factor (VF) genes, through mapping against the ResFinder (Florensa et al., 2022; https://bitbucket.org/genomicepidemiology/resfinder_db/downloads/) and Virulence Factor databases (Chen et al., 2005; https://www.mgc.ac.cn/VFs/download.htm), using Bowtie2 v. 2.2.9 (option: '–very-sensitive-local'; Langmead & Salzberg, 2012). Genes were grouped according to the antibiotic and virulence factor class they confer resistance to. Those genes conferring resistance to macrolides, lincosamides, streptogramins and pleuromutilins were merged into the MLSP class, while those that confer resistance to oxazolidinones into the oxazolidinone class as previously reported (Additional file 9 − Phenotypes table from Cobo-Díaz et al., 2021). Abundance matrices were transformed to count per million reads (CPM) matrices for further analyses using an R-script (https://github.com/JoseCoboDiaz/counts2CPM).

Metagenome assemblies were then used to perform a BLASTn v. 2.2.30 (Altschul et al., 1990) against the ResFinder and Virulence Factor databases using parameters '-max_target_seqs 1000′ and '-perc_identity 80′. Only contigs matching with identity and coverage ≥ 80 % were retained for further analyses and taxonomically classified with Kraken v. 2.0 (Wood et al., 2019), jointly with the "Standard plus protozoa & fungi (PlusPF)" database (available at https://benlangmead.github.io/aws-indexes/k2), using default parameters. The analysis of Mobile Genetic Elements (MGEs) was then performed using the assembled contig files as query files: plasmids were predicted by Plasflow ('threshold 0.7'; Krawczyk et al., 2018), lateral gene transfer (LGT) events were detected by WAAFLE (Hsu et al., 2023) and integrons were predicted by Integron_Finder (Cury et al., 2016).

Using their coordinates in the contigs, coding sequences (CDS) within LGT and integron regions were extracted from WAAFLE and Integron_Finder output files by using in-house ruby scripts and bedtools (Quinlan & Hall, 2010) utilities (https://github.com/JoseCoboDiaz/ARG-contig_ mobilome_analysis). The extracted CDS fasta files were used for BLASTn comparison against the ResFinder and Virulence Factor databases using an 80 % identity cut-off.

2.4. Statistical analysis

Data visualization and statistical analysis were performed in R environment (version 4.1.3; https://www.r-project.org).

Kruskal-Wallis and Wilcoxon rank sum tests ('kruskal.test' and 'wilcox.test' functions from the 'base' package) were used to assess significant differences in the abundance of taxa/genes between the groups and to compare alpha diversity indices, respectively, with a 0.05p-value threshold. Boxplots were drawn with functions 'geom_boxplot' and 'geom_jitter' from the 'ggplot2′ package.

The function 'vegdist' from the 'vegan' package was used to compute Bray-Curtis distances whereas 'geom_point' from 'ggplot2′ package plotted the first two Principal Coordinates. Heatmap plot was then produced using the function 'pheatmap' from the 'pheatmap' package.

Bubble plots and pie charts, used to show the distribution of AR and VF contigs in genera/species, were carried out using the functions 'geom_point' and 'geom_bar' + 'coord_polar', respectively from the 'ggplot2′ package.

Co-occurrence plots, used to display how many ARGs were present within each contig, were performed using the function 'geom_gene_arrow' from 'ggplot2′ package. The start and end locations of the genes within their contigs were mapped to the xmin and xmax aesthetics respectively.

Barplots figures were produced using the functions 'barplot' from the 'base' package, 'geom_col' and 'geom_bar' from the 'ggplot2′ package.

Finally, the function 'geom_line' from the 'ggplot2′ package was used to make a line plot, showing the most abundant VF genes (VFGs) for each group.

2.5. Data availability

The raw sequence reads generated in this study have been deposited in the Sequence Read Archive (SRA) of the National Center of Biotechnology Information (NCBI) under the accession number PRJNA1054326.

3. Results

3.1. Microbiome mapping along the beef processing chain

Microbial communities in the beef processing facility were highly complex and greatly differed in their structure. In particular, slaughterhouse surfaces hosted lower microbial diversity than carcass samples and retail butcher shop surfaces (Fig. 1). Moreover, samples from the retail butcher shop clustered apart from slaughterhouse surfaces and carcass samples (Fig. 2). The most abundant species in the beef processing facility were *Brochothrix thermosphacta*, *Carnobacterium maltaromaticum*, *Pseudomonas fragi*, *Psychrobacter cryohalolentis* and *Psychrobacter immobilis*, whose presence was found in all the processing areas (Fig. 2). *B. thermosphacta* and *Pseudomonas versuta* showed a higher abundance on retail butcher shop surfaces than on slaughterhouse surfaces, while *Psychrobacter cryohalolentis* and *Psychrobacter immobilis* were more abundant on slaughterhouse surfaces (Fig. 3).

For a deeper analysis, we performed MAGs-strain-level population analyses to assess strain relatedness between samples belonging to different processing areas and surfaces. From all the main species found, we only identified two clearly distinct strains for C. maltaromaticum, one specific for the butcher shop, while the second was shared between the three sample categories (carcasses, slaughterhouse surfaces and retail butcher shop) (Supplementary Figure S1).

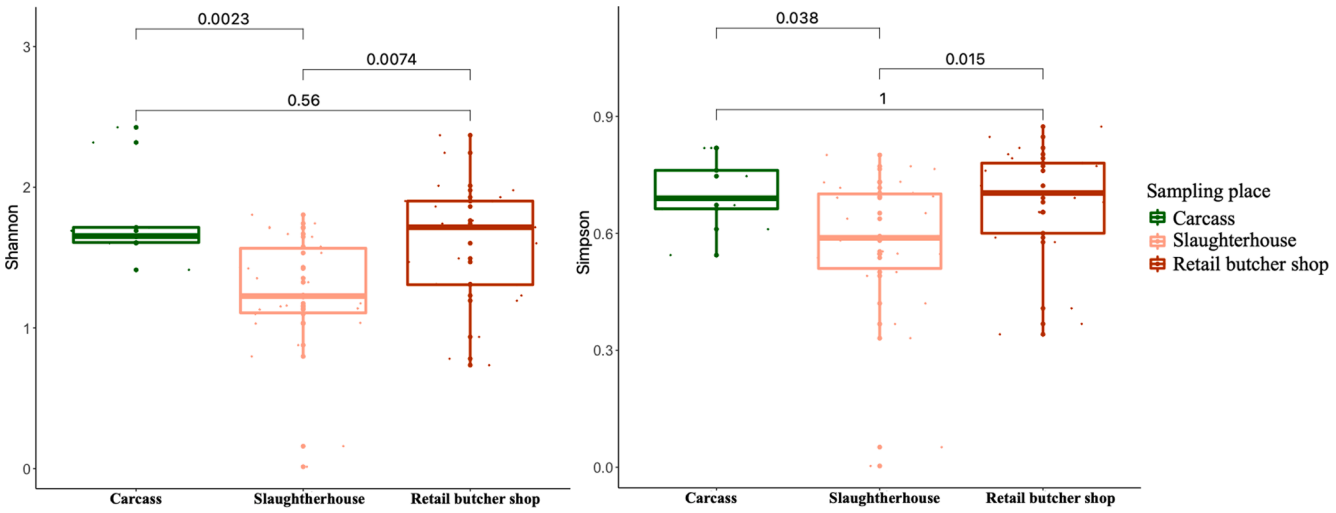

Fig. 1. Box plots showing the Shannon and Simpson diversity indices in swabs' samples taken along a beef processing facility. P-values were calculated using Wilcoxon Rank Sum Tests. Samples are grouped according to the processing areas: Green: Carcass; Pink: Slaughterhouse; Orange: Retail butcher shop. Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.5 IQR from the first and third quartiles, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

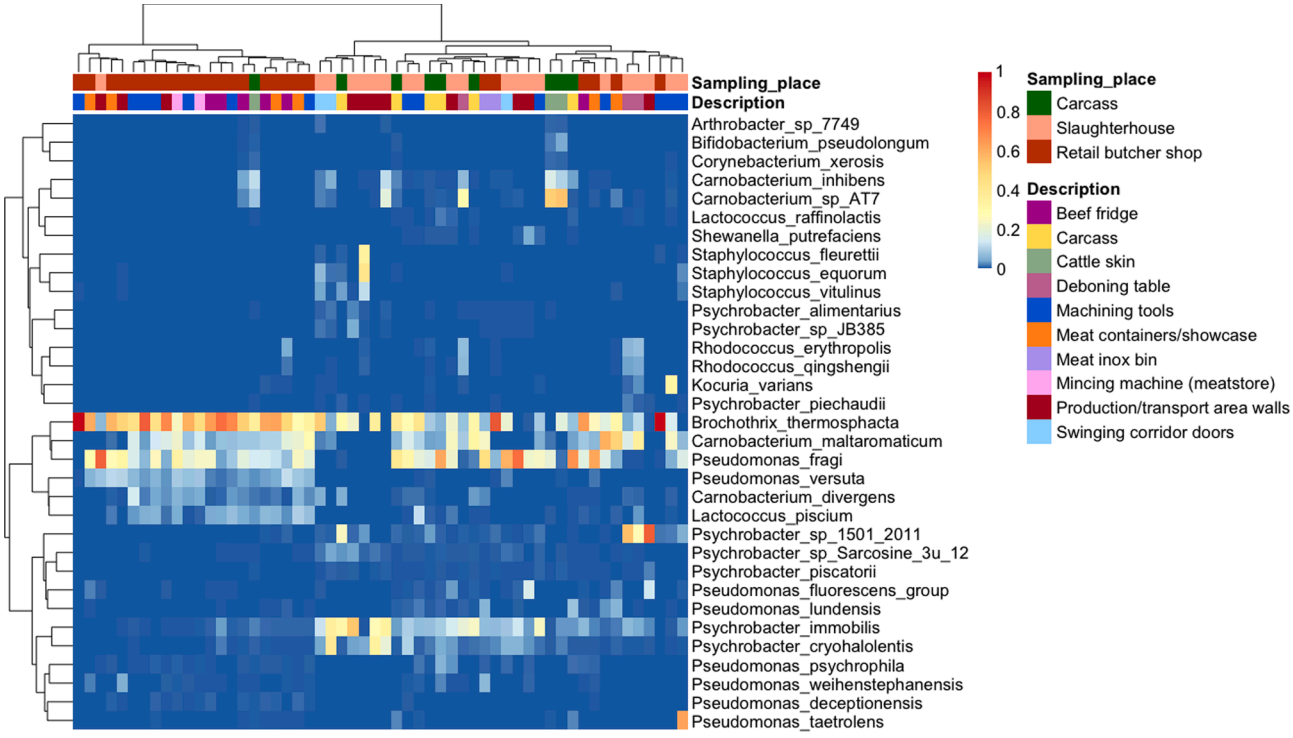

Fig. 2. Heat plot showing the abundance (%) of the most abundant microbial taxa in swabs' samples taken along a beef processing facility. Only taxa with abundances > 0.5 % are included. The column bars are color-coded according to the processing areas where they were collected (carcass at the beginning of the processing, slaughterhouse and retail butcher shop surfaces) and the type of sampled surface.

3.2. Packaging and temperature conditions during storage select for a microbiome with a different functional potential

A different microbiome composition was found in raw beef samples stored under different temperature and packaging conditions. In particular, samples stored at 0 °C were dominated by *Pseudomonas paraversuta*, *P. fragi*, *B. thermosphacta*, *Acinetobacter harbinensis*, *Ps. immobilis*, *Photobacterium carnosum* and *Leuconostoc gelidum* (Fig. 4A) whereas, in addition to the above-mentioned species, *Lactococcus paracarnosus*, *Lactococcus piscium* and *Latilactobacillus sakei* were more abundant in samples stored at 4 and 10 °C (Fig. 4B–4C). However, differences according to the packaging type were observed. AIR storage selected for different *Pseudomonas* species, such as *P. paraversuta*, *P. versuta* and *P. fragi* while *B. thermosphacta*, *A. harbinensis* and *Lc. gelidum* were more abundant in samples packed under MAP. Lactic acid bacteria (LAB) such as *Lc. piscium*, *Lc. paracarnosus*, *Carnobacterium divergens*, *Dellaglioa algida*, *Lc. carnosum* and *Lb. sakei* were identified as main SSOs in samples packed under VP (Figs. 4-5A).

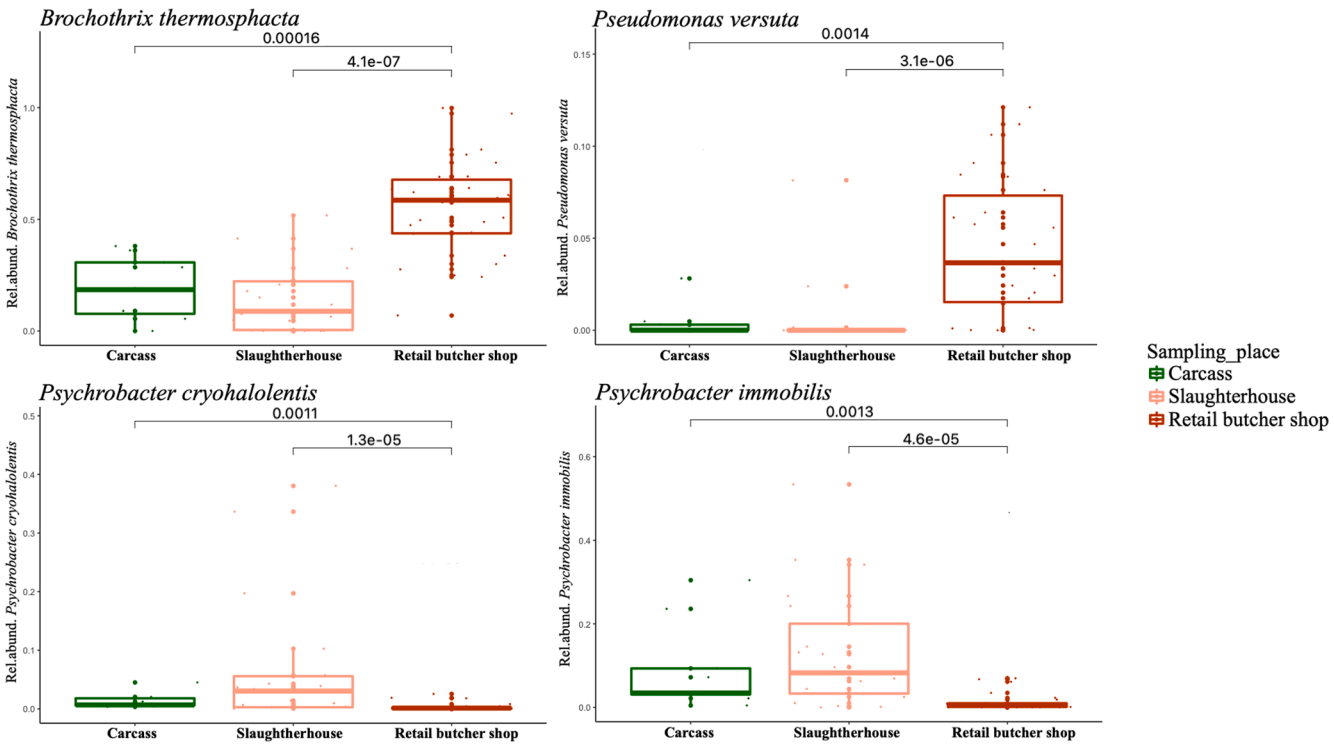

Fig. 3. Box plots showing the relative abundance of *Brochothrix thermosphacta*, *Pseudomonas versuta*, *Psychrobacter cryohalolentis* and *Ps. immobilis* in swabs' samples taken along a beef processing facility. Samples are grouped according to the processing areas. P-values were calculated using Wilcoxon Rank Sum Tests and only those significant ($p \leq 0.05$) are shown.

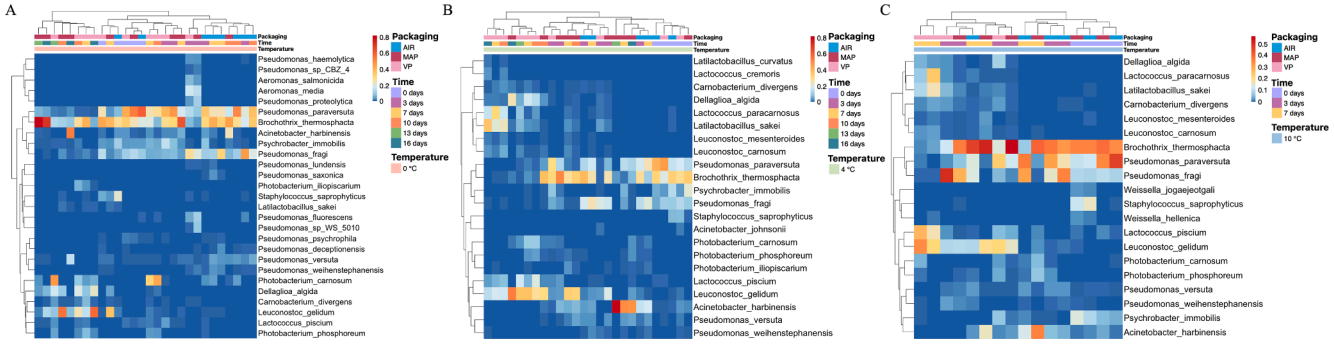

Fig. 4. Heat plot showing the abundance (%) of the most abundant microbial taxa in the raw beef samples analyzed. Only taxa with abundances > 1 % are included. The column bars are color-coded according to the packaging conditions, the different time points and storage temperature. A: Raw beef samples stored at 0 °C; B: Raw beef samples stored at 4 °C; C: Raw beef samples stored at 10 °C.

We also investigated the functionality of the microbiome in relation to the potential production of different VOCs associated with meat spoilage. Several genes coding for acetoin/diacetyl, 2,3-butanediol, acetaldehyde and acetate production, off-odor compounds coming from carbohydrate metabolism and often reported in spoiled meat, were found in all the samples and their abundance was higher in raw beef stored under MAP and VP conditions (Fig. 5B). In contrast, different genes involved in amino acid and protein degradation showed a greater abundance in beef samples stored in AIR packaging (Fig. 5C).

Finally, we highlighted a distinct distribution of ARGs. Samples stored in AIR packaging harbored a lower diversity of antibiotic families, which decreases over time. On the contrary, VP samples stored at 0 and 4 °C maintained high diversity over time suggesting that lower temperatures may select species with a broader pattern of ARGs (Supplementary Figure S2).

3.3. Antibiotic resistance and virulence factors along the beef production chain

Reads from the beef production chain were screened for the presence of Antibiotic-Resistance (AR) and Virulence Factors (VF) genes to understand the potential risks associated with their presence. A preliminary examination revealed an interesting pattern: carcass samples harbored a greater amount of ARGs (Fig. 6A) while VFGs were instead enriched on retail butcher shop surfaces (Fig. 6B). More in detail, the most abundant AR classes were associated with resistance to aminoglycosides, beta-lactams, MLSPs (class including genes conferring resistance to macrolides, lincosamides, streptogramins and pleuromutilins), amphenicols, sulfonamides, tetracyclines and oxazolidinones, with MSLPs being exclusively detected on carcass samples and slaughterhouse surfaces and oxazolidinones found only on slaughterhouse surfaces (Fig. 6A). Adherence, biofilm, effector delivery system, motility, nutritional/metabolic factors and regulation were instead the

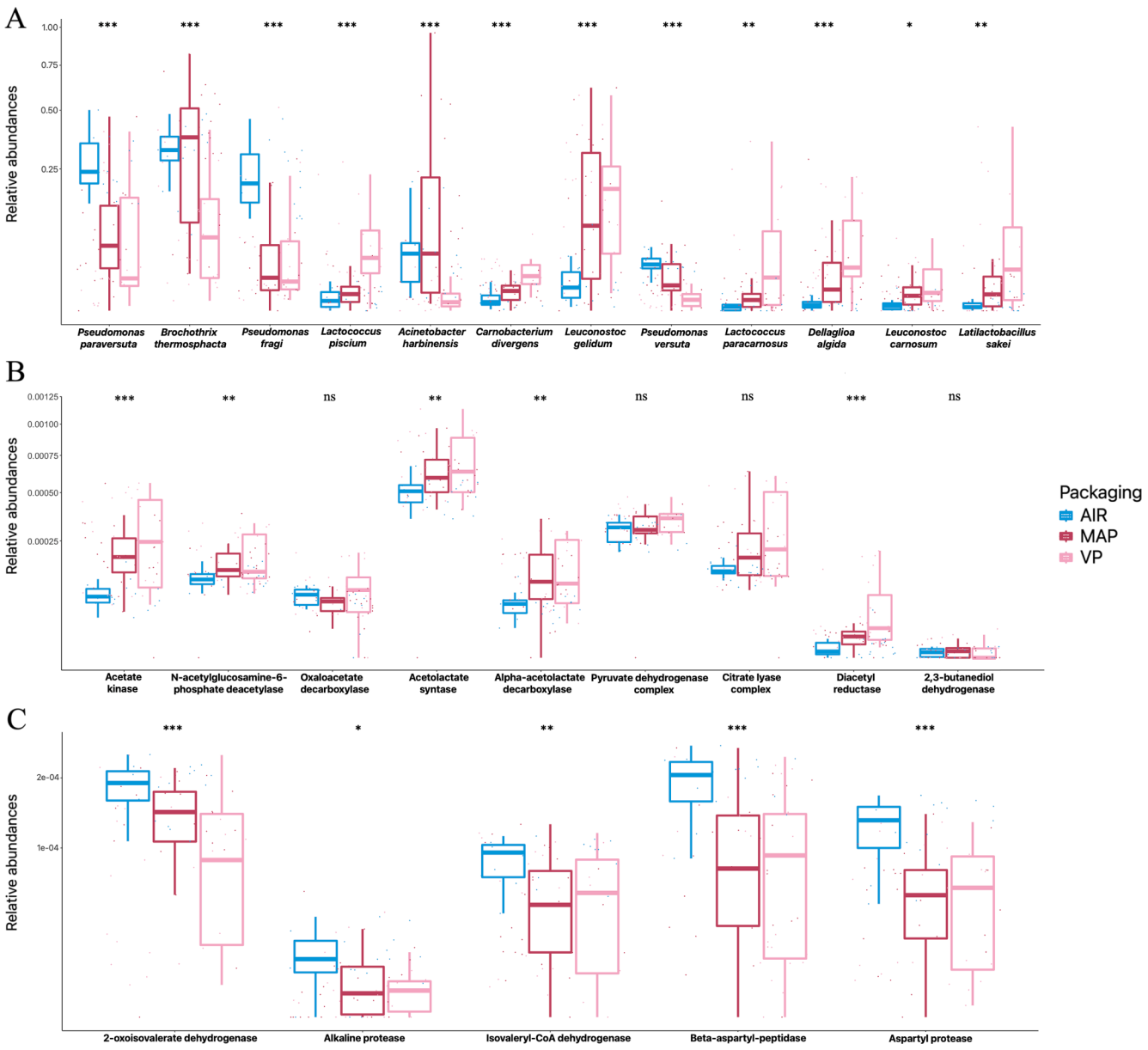

Fig. 5. Box plots showing the relative abundances of dominant raw beef spoilage bacteria (A) and of genes involved in acetoin/diacetyl and acetate production (B) and amino acid and protein degradation (C) in the different packaging conditions. P-values were calculated using Kruskal–Wallis Tests. Significance codes: '***' p-value between 0 and 0.001, '**' p-value between 0.001 and 0.01, '*' p-value between 0.01 and 0.05, 'ns' not significant.

predominant VFs, all present at higher levels on retail butcher shop surfaces (Fig. 6B). Accordingly, higher abundance of biofilm and motility genes on retail butcher shop surfaces was also highlighted (Fig. 7).

Several different VFs belonging to effector delivery systems (*vgrG1b*, *tssH*, *tssF*, *tssE*, *tssC*, *icmH/tssL*, *hsiC1/vipB/tssC*, *hsiB1/vipA/tssB, hcpA*, *clpV1),* regulation (*sigA/rpoV*, *rpoS*), adherence (*rpoN*, *pilT*, *pilJ*, *pilG*, *ompA*, *crc*), nutritional/metabolic factors (*pvdS*, *pvdL*, *pvdH*, *fpvA*, *fepA*), motility (*PA1464*, *PA1459*, *PA1458*, *motC*, *fliP*, *fliN*, *fliI*, *fliG*, *fliF*, *fliA*, *flhA*, *flgI*, *flgH*, *flgG*, *flgC*, *fleQ*, *fleN*) and biofilm (*mucP, mucD, algW, algI, algD, algA, alg8*) were among the most abundant VFGs found in swabs' samples taken along the beef processing chain. As shown, the profiles of the different processing areas were quite similar but the abundance of these genes was higher on retail butcher shop surfaces, highlighting that this environment can be considered a reservoirs of these genes (Supplementary Fig. 3).

Considering the overall quantity of ARGs, the highest concentrations were observed on the cattle carcasses but there was no difference between the different surfaces belonging to the same sampling area (Supplementary Fig. 4).

Furthermore, in order to deepen and complete the analysis, also metagenome assemblies were screened for the presence of ARGs and VFGs. Results obtained in this context highlighted that, among the AR classes, aminoglycosides were the most widespread (273 occurrences), followed by tetracyclines (256), folate pathway antagonists (84), beta-lactams (41) and amphenicols (39), accordingly with what was observed in the read-level analysis, except for folate pathway antagonists (Fig. 8A). We also assessed the contribution of each taxon to the number of AR occurrences: overall, 142 ARGs were attributed to *Acinetobacter* spp., 135 to *Psychrobacter* spp., 42 to *Staphylococcus* spp., 37 to *Moraxella* spp. and 33 to *Enterococcus* spp. Interestingly, 170 ARGs were labelled as 'Unclassified' (Fig. 8A). Furthermore, *Acinetobacter* contributed the most to the spread of tetracyclines, with 51 occurrences overall, followed by *Psychrobacter* and *Staphylococcus*. *Psychrobacter* also carried several

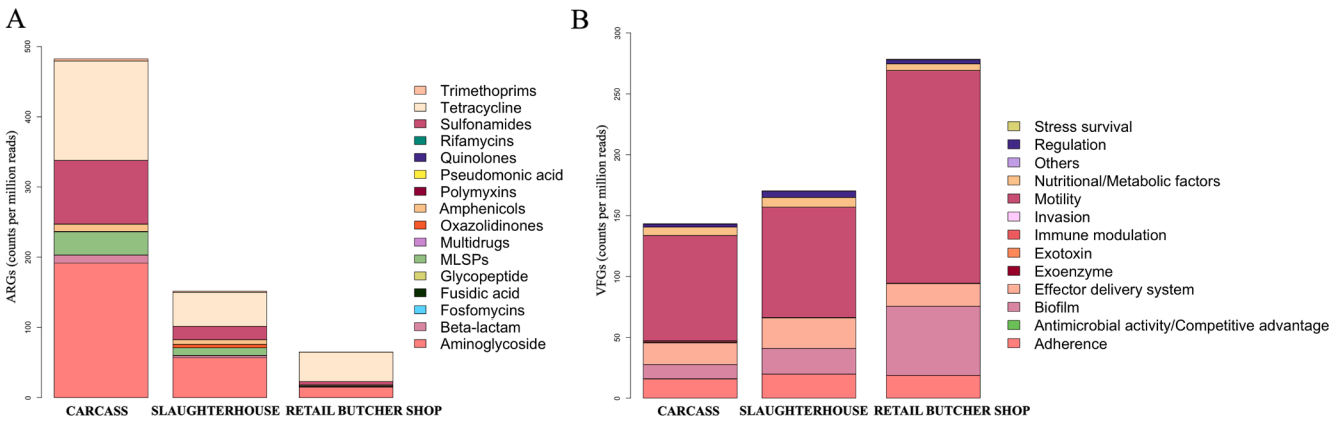

Fig. 6. Barplot showing the abundance in counts per million reads (CPM) of the Antibiotic Resistance Genes (A) and Virulence Factor Genes (B) classes. Each bar represents the average value for swabs' samples belonging to the same processing area. MLSPs refers to the sum of macrolides, lincosamides, streptogramins and pleuromutilins antibiotic classes.

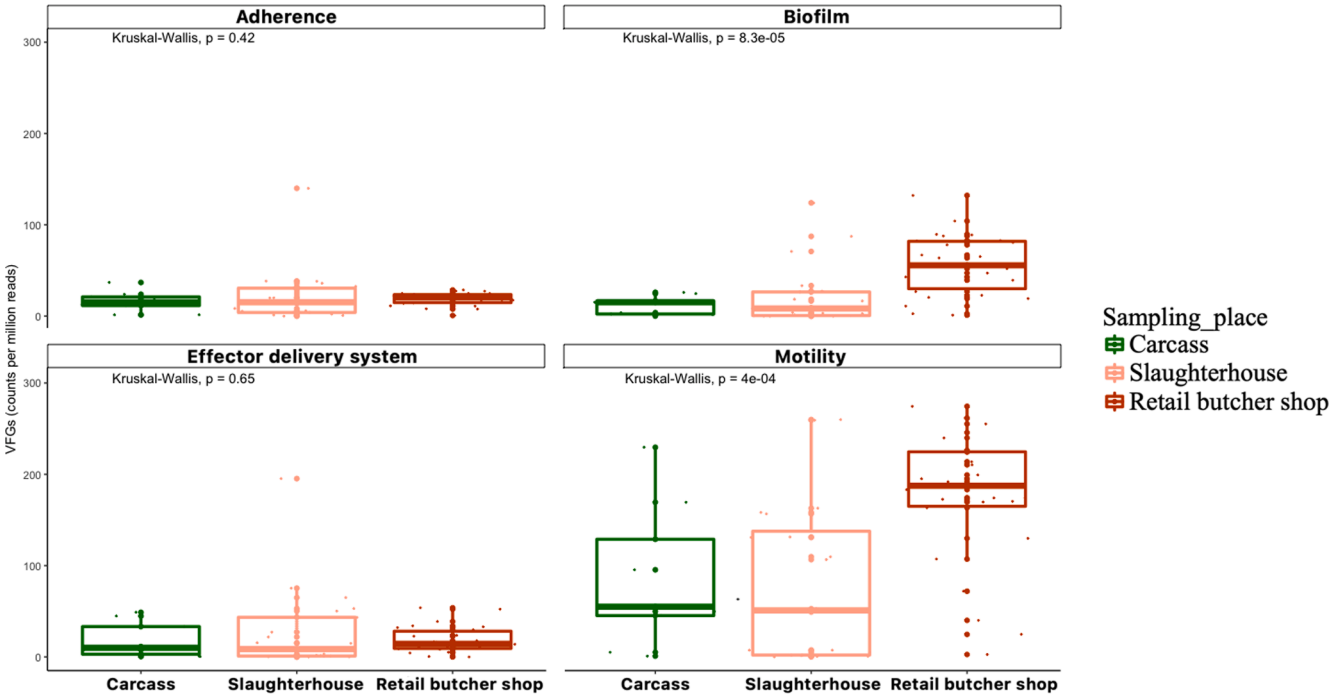

Fig. 7. Box plots showing the abundance in counts per million reads (CPM) of the main virulence factors (adherence, biofilm, effector delivery system and motility) in swabs' samples taken along beef processing facility. Samples are grouped according to the processing areas. P-values were calculated using Kruskal–Wallis Tests.

genes conferring resistance to aminoglycosides (n = 66), followed by *Acinetobacter* (n = 44), *Moraxella* (n = 23), *Staphylococcus* (n = 17) and *Enterococcus* (n = 15) (Fig. 8A). Consistently, *Acinetobacter* and *Psychrobacter* spp. were the most important contributors to ARGs in all the surfaces sampled (Fig. 8B).

Among the VFs, motility was the most widespread (1,760 occurrences), followed by effector delivery systems (704), adherence (618), biofilm (205), nutritional/metabolic factors (191) and immune modulation (79) (Supplementary Fig. 5). Also in this case, we evaluated the contribution of each taxon to the number of VFs occurrences: overall, 918 VFGs were attributed to *P. fragi*, 342 to *P. lundensis,* 284 to *P. versuta* and 251 to *P. psychrophila* (Supplementary Fig. 5). P. *fragi* contributed the most to the spread of motility genes, with 618 occurrences overall, followed by *P. versuta*, *P. psychrophila* and *P. lundensis*. *P. fragi* also carried several adherence-related genes (n = 201), followed by *P. psychrophila* (n = 45), *P. versuta* (n = 28) and *P. lundensis* (n = 21). On the contrary, VFs related to effector delivery systems (n = 143) were mostly assigned to *P. lundensis* (Supplementary Fig. 5).

3.4. Beef chain microbiome hosts antibiotic resistance genes on mobile elements

In order to understand whether the resistome can be mobilized, we screened the ARG-carrying contigs for the presence of mobile elements, using 3 different tools (Plasflow, WAAFLE, Integron_Finder). We computed the percentage of positive calls for each class of genes according to the results from each tool (Supplementary Fig. 6A). Beta-lactams genes were frequently associated to plasmids (78.05 % of the genes according to Plasflow), together with lincosamides (70.59 %), aminoglycosides (69.60 %), amphenicols (69.57 %), macrolides (58.49 %), tetracyclines (52.34 %) and folate pathway antagonists genes (46.59 %). Also, 7.55 % of the macrolides, 4.54 % of the folate pathways antagonists, 2.44 % of the beta-lactams and 1.96 % of the lincosamides resistance genes were predicted to be involved into LGT events. Finally,

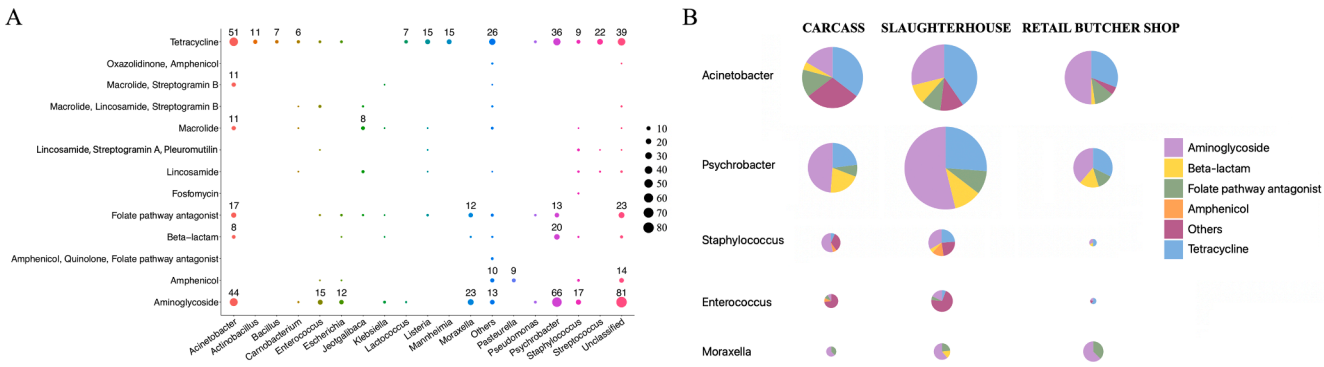

Fig. 8. Bubble plot showing the number of antibiotic resistance occurrences attributed to each taxon. 'Others' were used to label all the genera that have < 5 antibiotic resistance contigs (A). Pie charts showing, for each processing area, the contribution of each genus in terms of antibiotic resistance classes. Slices are color-coded according to the different antibiotic resistance classes. 'Others' were used to label all the antibiotic resistance classes that have < 35 antibiotic resistance contigs. The size of the circle is proportional to the contribution of each genus to each area (B).

15.75 % of the aminoglycosides, 13.64 % of the folate pathways antagonists, 6.52 % of the amphenicols and 2.44 % of the beta-lactams resistance genes were predicted to be integrons. Furthermore, we checked whether some genes co-occurred on the same contigs, and we found 8 contigs hosting at least 3 ARGs (Supplementary Fig. 6B). In particular, we observed that aminoglycosides resistance genes (*aac(3)-IId*, *aac(6′)-IIa*, *ant(3″)-Ia*, *ant(6)-Ia*, *aph(3″)-Ib*, *aph(3′)-Ib*, *aph(6)-Id*) usually co-occurred together and/or with folate pathway antagonists (*sul1* and *sul2*), amphenicols (*catB3*) and tetracyclines (*tet(H)*, *tet(Y)*) ARGs. However, although we only focused on contigs showing at least 3 ARGs, more contigs showing multiple genes were identified, with 119 contigs harboring at least 2 ARGs. Interestingly, most of the contigs showing co-occurrence of genes were predicted to be part of plasmids.

4. Discussion

In this study, we monitored the microbiome of processing environments along a beef processing chain, from the slaughterhouse to the retail shop. Slaughterhouse and butcher shop processing environments are considered as a primary source of contamination for raw meat (De Filippis et al., 2013; Bughti et al., 2017). Consistently with previous reports, we found high microbial diversity on beef processing surfaces. Although the main taxa present in the environment are the same, their abundance differed in the two processing areas (slaughterhouse and butcher shop), leading to a separate clustering of the samples. This suggests that these environments are colonized by a complex and psychrotrophic core microbiome, that is well adapted to the meat-processing conditions and nutrients (e.g., low temperature, meat exudates), although the microenvironments specific of the two areas may lead to different patterns of abundance.

Despite the high complexity of the environmental microbiome, *B. thermosphacta* and other psychrotrophic genera such as *Pseudomonas*, *Psychrobacter* and *Carnobacterium* are identified as the most abundant taxa that characterize beef processing, as previously reported (Wagner et al., 2020; Maes et al., 2019). In particular, *B. thermosphacta* is known as a facultative anaerobic spoiler of raw meat and was previously described to be present on surfaces in meat-handling environments (Casaburi et al., 2014; Maes et al., 2019; Møretrø & Langsrud, 2017; Quijada et al., 2018), together with *P. fragi* and *P. versuta*, often reported as part of the residential microbiome of food processing environments (Zwirzitz et al., 2020; Røder et al., 2015). *Psychrobacter* and *Carnobacterium* spp. have been often identified in meat processing environments, including sausage processing plants (Hultman et al., 2015), slaughterhouses (Røder et al., 2015; De Filippis et al., 2013), butchers' shops (Stellato et al., 2016) and air and different processing sites in a beef processing facility (Fagerlund et al., 2017). These findings support the hypothesis that these taxa are part of a core microbiome shared by all meat-handling facilities and that they may be transferred from surfaces to meat during processing and handling, where they may proliferate and lead to spoilage.

Furthermore, we highlight that different packaging and storage conditions can strongly affect raw beef microbiome during the shelf-life, as well as its functional potential. Samples stored in AIR packaging exhibited a higher abundance of various *Pseudomonas* species, including *P. paraversuta*, *P. versuta*, and *P. fragi*. In particular, *Pseudomonas* spp. produce proteolytic enzymes involved in food spoilage (Stanborough et al., 2018) that causes undesirable changes in meat, such as breaking down of proteins and production of a variety of off-odors (Gram et al., 2002). *Pseudomonas* spp., particularly *P. fragi*, show a wide metabolism and can produce several types of VOCs impacting on the sensorial profile (Ercolini et al., 2010). However, strain-level differences in the potential metabolic activity (De Filippis et al., 2019; Papadopoulou et al., 2020; Stellato et al., 2017) and in the VOC pattern (Ercolini et al., 2010; Pavlidis et al., 2021) have been reported.

On the other hand, samples packed under MAP showed a higher abundance of *B. thermosphacta, Leuconostoc gelidum* and *Acinetobacter harbinensis*. On the contrary, LAB, such as *Lactococcus piscium*, *Lc. carnosum, Lc. paracarnosus*, *Carnobacterium divergens*, *Dellaglioa algida*, and *Latilactobacillus sakei* were identified as the main spoilage taxa in samples packed under VP. *B. thermosphacta* is a facultative-anaerobic taxon for which meat is considered an ecological niche and it is able to produce off-flavours from carbohydrates degradation (Casaburi et al., 2015). *Dellaglioa algida*, originally described as *Lactobacillus algidus* and recently reclassified (Zheng et al., 2020), has been described as animal-associated and highly abundant in a variety of chilled-stored, vacuum-packed and MAP meat products (Werum & Ehrmann, 2024). In addition, *D. algida* in VP beef is considered a protective species, preventing the growth of other meat spoilers and, thereby, reducing the production of spoilage-associated volatile compounds (Mansur et al., 2019; Pavlidis et al., 2019).

The interactions among microbiome members lead to the production of several types of VOCs, including ketones, aldehydes, esters and alcohols that contributes to the development of meat unpleasant odors (Mansur et al., 2019; Casaburi et al., 2015). We identified genes involved in the production of off-flavours from carbohydrates in all the samples, with higher levels in MAP and VP whereas genes associated with amino acid and protein degradation were enriched in AIR. Indeed, this may be due to a delayed microbial growth in MAP and VP, where residual carbohydrates are found, as previously suggested (De Filippis et al., 2019). Acetoin and diacetyl produce buttery, creamy and cheesy odors, that can give unpleasant odor to fresh meat, that is regarded as "not fresh" (Casaburi et al., 2015). However, VOCs produced from carbohydrates usually have a lower sensorial impact compared to those arising from amino acid and protein degradation (Casaburi et al., 2015).

In addition, our results revealed a distinct distribution of ARGs in samples stored in different packaging and temperature conditions and suggests that lower temperatures may select species with a broader pattern of ARGs.

Finally, we screened meat processing metagenomes for the presence of AR and VF genes and a diverse array of these genes was found along the beef processing chain. Carcass samples exhibited high levels of ARGs mainly associated with resistance to aminoglycosides, beta-lactams, MLSPs, amphenicols, sulfonamides, tetracyclines and oxazolidinones antibiotic classes, consistently with what has been previously reported (Cobo-Díaz et al., 2021; Cameron et al., 2016).

In contrast, retail butcher shop surfaces hosted a higher concentration of VFGs. In particular, adherence and effector delivery system associated genes showed a greater abundance on slaughterhouse surfaces, while biofilm and motility genes were instead enriched on retail butcher shop surfaces.

Interestingly, we observed a high amount of ARGs on processing surfaces, where the production of biofilms may enhance the acquisition of resistance (Carrascosa et al., 2021). Biofilm production is an excellent survival strategy, since bacteria are protected from antimicrobials and only exposed to sub-Minimum Inhibitor Concentrations (MIC) of such compounds (Zhang et al., 2020). The bacterial ability to adhere and form biofilms on biotic and abiotic surfaces causes an increase in virulence, as well as in pathogenicity. Bacterial attachment is facilitated through cell surface organelles, such as flagella, pili, fimbriae and curli (Schroeder et al., 2017). It is well known that *Brochothrix*, *Pseudomonas*, *Psychrobacter*, *Acinetobacter* and *Staphylococcus* spp. are able to produce biofilm in the food industry (Casaburi et al., 2014; Souza et al., 2014; Nikolaev et al., 2022; Wagner et al., 2020). In addition, the use of some compounds (such as disinfectants) to combat biofilms might enhance antibiotic resistance through a mechanism named cross-resistance (Colclough et al., 2019; Kampf, 2018). For all these reasons, biofilms may enhance ARG development and transmission (Uruén et al., 2020), therefore food industries should develop alternative methods to avoid their attachment and maturation.

We also highlighted that most of the AR genes belonged to *Acinetobacter*, *Psychrobacter* and *Staphylococcus* spp. This result is not surprising as several authors reported the AR potential of these three taxa in food industry surfaces. For example, *Acinetobacter* has been often associated with dairy products and meat (Gurung et al., 2013; Ramos et al., 2019; Klotz et al., 2018) and, although *A. baumannii* is the principal pathogenic species described, AR has been reported for the whole genus (Crippen et al., 2020). Our results suggested that *Acinetobacter* spp., more abundant on carcass samples and slaughterhouse surfaces, were strongly linked with resistance to tetracyclines. On the contrary, *Psychrobacter* spp. were predominant on the retail butcher shop surfaces, mainly contributing to aminoglycosides resistance.

Our analysis also highlighted a wide range of potential VFs, mainly associated with *Pseudomonas* spp. and related to motility, effector delivery systems, adherence, biofilm, nutritional/metabolic factors and immune modulation. *Pseudomonas* spp. have been widely reported as common inhabitants of food-handling environments (Valentino et al., 2022; Stellato et al., 2016) and their adaptation to environmental stress through the production of biofilms has been widely described (Fazli et al., 2014; Mann & Wozniak, 2012). In addition, biofilms produced by *Pseudomonas* may potentially entrap pathogenic microbes, thus protecting them from external stress (Caraballo Guzmán et al., 2020).

Finally, our results suggested that a high percentage of beta-lactams, lincosamides, aminoglycosides, amphenicols, macrolides, tetracyclines and folate pathway antagonist resistance genes were encoded in plasmids or other mobile elements, and often co-occur on the same contig. Even though these genes are not linked to pathogenic taxa, the extensive mobilization of ARGs demands attention. It has been proposed that sharing the same ecological habitat is a crucial factor in regulating LGT events (Smillie et al., 2011). Consequently, as food contaminated with AR species passes through the gastrointestinal tract, LGT events may occur, even with phylogenetically distant species, as previously reported (Rolain, 2013). However, it is important to underline that LGT events occurs very rarely and it may only partially contribute to the spreading of resistant pathogens. In addition, the presence of mobile elements on surfaces and in foods raises concerns, as they may facilitate the simultaneous transmission of multiple AR genes, conferring resistance to various antibiotic classes to microorganisms commonly associated with food consumption.

5. Conclusion

Our exploration of the microbiome composition, antibiotic resistance, and virulence potential in a beef processing chain, integrated with the study of the influence of various packaging and storage conditions of raw beef, reveals the intricate interplay of factors that significantly influence meat safety and quality. Notably, the distinct microbial community structures found in the different beef processing areas and surfaces highlights the importance of microbiome-mapping procedures to identify specific contamination routes and potential risks for meat quality and safety. However, we must underlie that each beef chain may have a specific microbial community and its dynamics. Therefore, these results should not be generalized, but may represent an important contribution to understand microbial patterns along food chains.

CRediT authorship contribution statement

Giuseppina Sequino: Writing – original draft, Formal analysis, Data curation. **José F. Cobo-Diaz:** Writing – review & editing, Formal analysis, Data curation. **Vincenzo Valentino:** Writing – review & editing, Formal analysis. **Chrysoula Tassou:** Formal analysis. **Stefania Volpe:** Formal analysis. **Elena Torrieri:** Writing – review & editing, Supervision. **George-John Nychas:** Writing – review & editing, Funding acquisition, Conceptualization. **Avelino Álvarez Ordóñez:** Writing – review & editing, Supervision. **Danilo Ercolini:** Writing – original draft, Funding acquisition, Conceptualization. **Francesca De Filippis:** Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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

I have shared my data in the section 'Data availability' of the manuscript.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114318.

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