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Microbiome and resistome successions in pig carcasses and fresh pork meat throughout slaughtering, processing and shelf-life

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Abstract

Background Slaughterhouses and meat cutting plants represent potential hotspots for the spread and transfer of spoilage and pathogenic, including antimicrobial resistant, bacteria to meat and meat products. Here, we characterise the progression of the microbiome and resistome of two pork cuts (loin and sirloin) at different stages of processing, from the slaughter line to the end of shelf-life. To this end, we analysed samples from facility surfaces, carcasses, and meat cuts using whole metagenome sequencing.

Results The taxonomic and antimicrobial resistance gene (ARG) profiles of carcasses and meat cuts were significantly influenced by the point of sampling and the processing room. The facility surfaces were found to be the main source of some abundant genera, such as *Anoxybacillus, Acinetobacter*, *Pseudomonas*, and *Brochothrix*, in carcasses and meat cuts. A total of 1,291 metagenome-assembled genomes were reconstructed, corresponding to the most prevalent species identified in the taxonomic analysis at the read level. A reduction in bacterial and ARGs richness and diversity was observed for carcasses and meat cuts along the production chain, which suggests that processing procedures are effective in reducing bacterial and ARGs loads. Nonetheless, an increase in the ARGs load was observed at two sampling points: the carcass after evisceration and the sirloin at the end of its shelf-life (in this case linked to the increase of a single gene, *tet(L)*). The ARGs most frequently detected were those associated with resistance to tetracyclines, aminoglycosides, and lincosamides. *Acinetobacter* (in processing environments and carcass/meat samples) and *Staphylococcus* (in carcasses and meat) were identified as the main genera associated with the ARGs found.

Conclusions Overall, our results provide the most detailed metagenomics-based perspective on the microbial successions of pig carcasses and fresh meat cuts during slaughtering, processing, and commercialisation. The observations made suggest that selection pressures imposed by processing steps and contact with facility surfaces contribute to shaping the microbiome and resistome of the two pork products throughout their production line and shelf-life.

Keywords Antimicrobial resistance genes, Pork meat, Carcass, Metagenomics, Resistome, Microbiome, Processing environments

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Background

The food industry is actively engaged in efforts to minimise the risk of pathogenic and spoilage bacteria during food production and processing. This is of particular importance for the meat production industry, as meat provides an optimal environment for microbial growth and the development of spoilage bacteria due to its high nutrient availability and water activity [1–3]. Slaughterhouses and meat cutting plants represent potential vectors for the spread and transfer of pathogens and spoilers to final products via raw materials or cross-contamination through facility surfaces and hygiene breaches. In this sense, food contact surfaces from meat facilities are important areas where microorganisms can persist and multiply [4, 5]. In addition, surfaces that do not come into direct contact with food can also be potential reservoirs of microbes and act as sources of contamination [6]. Moreover, bacteria that colonise meat processing environments can play an important role in the transmission of antimicrobial resistance genes (ARGs), posing an additional risk to human health [6–9]. The World Health Organization has identified antimicrobial resistance (AMR) as one of the top 10 global public health threats [10] and the potential contribution of the food chain is an emerging concern. The occurrence and spread of antimicrobial resistant microorganisms in the food chain can be promoted through the misuse of antimicrobials in food-producing animals. However, the extent to which the food chain contributes to the global burden of AMR spread remains unknown [7, 8, 11].

Mapping the routes of entry and establishment of microorganisms, including antimicrobial resistant bacteria, in meat production lines has been challenging, mainly due to the limitations of culture-dependent methods. While these techniques are effective for identifying and quantifying the distribution of specific bacterial groups of interest, they do not allow untargeted analyses of complex microbial communities [12]. The recent emergence of methods based on Next Generation Sequencing has provided new means for mapping microbial ecosystems prevailing in the food industry [12–14]. Metataxonomic approaches, such as those based on the sequencing of *16S rRNA* gene amplicons, have recently been used to determine the sources of contamination and transmission routes for microbial populations throughout meat processing facilities [15, 16]. Nevertheless, certain issues associated with this methodology were identified, including potential biases due to differential amplification of certain taxa [13, 14]. Conversely, whole metagenome sequencing (WMS) analyses entail the untargeted sequencing of all genetic material from a microbial community, thereby offering a valuable means of overcoming the limitations of traditional methods [12, 14, 17]. Besides the analysis of the taxonomic profile of the microbiota, this approach allows the characterisation of the genomic content, facilitating the analysis of a broad spectrum of genes of interest, such as ARGs, within the whole metagenome, and even the reconstruction of metagenome-assembled genomes (MAGs) [17, 18].

The large number of studies using WMS in human microbiome research or clinical settings [19–22] contrasts with the scarce use of this technology in food industries [23–25], and particularly within the meat sector [6, 26]. In relation to the resistome, some authors have conducted studies within meat industries, using samples obtained from slaughterhouses or cutting plants, including fecal, surface, and meat samples [6, 27, 28]. However, a comprehensive analysis through WMS of the metagenome and resistome of a meat product and its associated processing environment throughout the production line, from slaughter to the end of the product's shelf-life, has not yet been conducted.

The current study aimed to assess the dynamic changes in the microbiome and resistome of fresh meat throughout the processing line and shelf-life, in order to ascertain the influence of farm of origin and of the different food processing steps and environments as microbiome sources. Towards achieving that aim, pig carcasses and two meat cuts (loin and sirloin) were monitored through WMS from the start of the slaughter line to the end of the meat cuts' shelf-life. Additionally, the microbiome and resistome of associated processing environments from the slaughter line, cooling rooms, and cutting plant were also characterised.

Materials and methods

Facility layout and sampling plan

The research was carried out at a Spanish pork processing facility where the slaughter line and cutting plant are physically connected. The industry processes around 70,000 pigs per week, equivalent to approximately 700 carcasses per working hour in the slaughter line. Due to the impossibility of stopping the processing line for sampling, two persons from our laboratory were placed at each sampling point in locations that did not interfere with the slaughterhouse or cutting plant operations, always under the supervision of staff from the industry's quality department. Figure 1 illustrates the workflow of the slaughterhouse and cutting plant, and shows the sampling points included in this study. Within the carcass processing area, scalding (by steam tunnel), de-hairing, singeing, polishing, and carcass splitting are automatic activities performed by machines/robots while other activities such as evisceration and fat removal are performed manually. After post-mortem inspection, the carcasses are stored overnight in the cooling room at 2 °C.

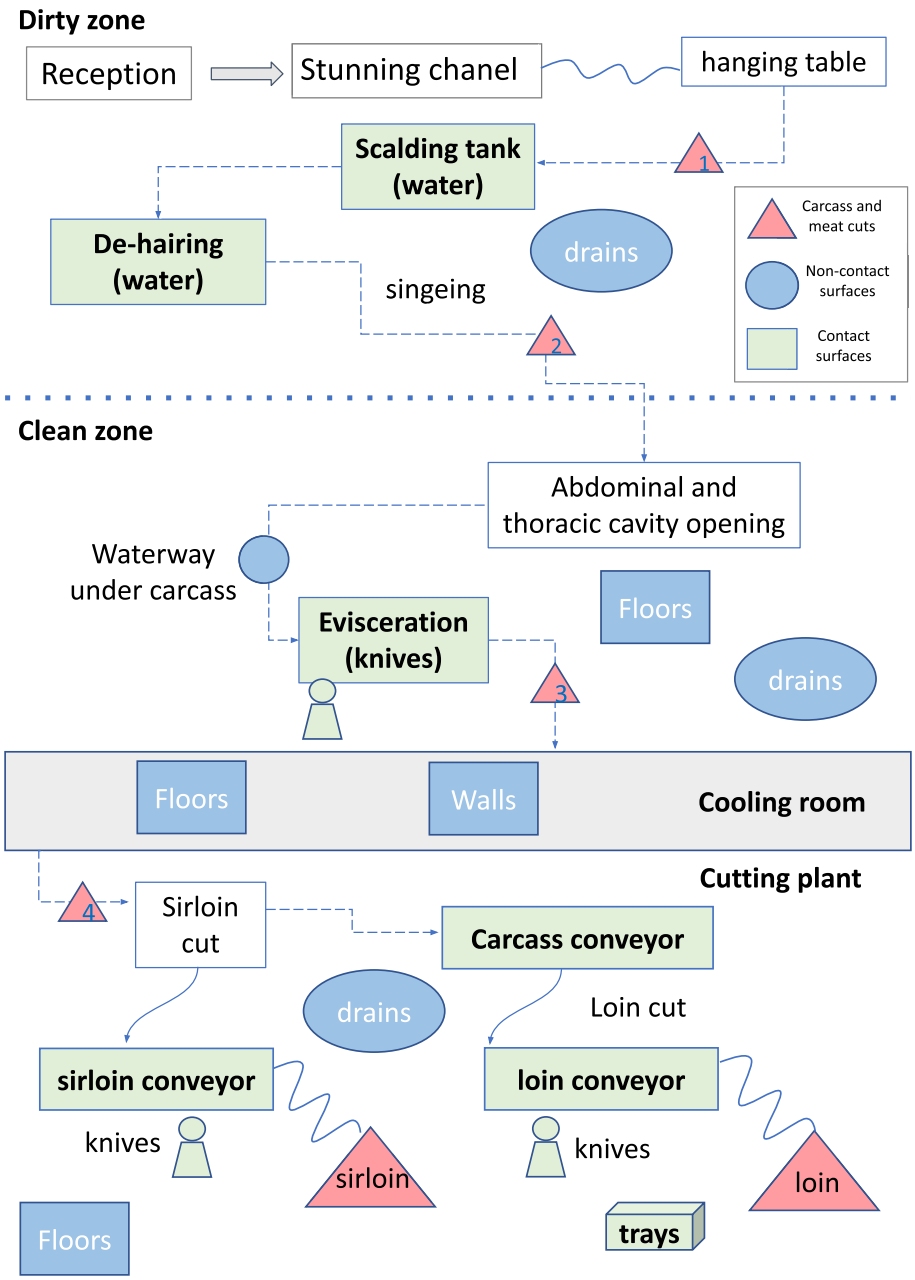

Fig. 1 Schematic layout of the whole processing line, including the slaughterhouse, cooling room and cutting plant. Green boxes indicate the contact surfaces that were sampled. Blue circles or boxes indicate non-contact surfaces sampled (floors, walls and drains). Red triangles indicate carcass samples taken at different stages of processing (1 = carcass start, 2 = carcass intermediate, 3 = carcass final, and 4 = carcass cooling), as well as sirloin and loin samples

On the following morning, the carcasses are moved by the automatic rails into the cutting plant for processing. There, carcasses are processed into the different cuts by operators. In this study, we selected the loin and sirloin production lines for analysis. In the initial stage of processing, sirloins were removed from half carcasses to a specific line for their fabrication, while the remaining half carcasses were placed on conveyor belts for larger cuts. After several cuts, the loins were removed and moved to specific conveyors for product preparation.

Considering the scheme detailed above, the sampling was conducted over two consecutive days. On the first day, samples were collected from both carcasses and environmental surfaces along the slaughter line. Twenty

carcasses from four different farms (five pigs per farm) were monitored at four sampling points within the slaughter line (Fig. 1 and Table S1); these carcasses were marked with zip ties on one of the front legs to follow them along the processing line, and they were constantly monitored and followed by staff of the industry's quality department to avoid mistakes in sampling different carcasses than the ones selected. Carcasses from Farm 1 and Farm 2 were processed at the beginning of the working day (designated as Time 1, T1, in the manuscript), while those from Farm 3 and Farm 4 were processed 4 h after the beginning of the working day (Time 2, T2). In addition, samples from drains, de-hairing equipment, and the scalding tank from the dirty zone ($n=12$), and samples from knives, drains, floors, and waterways under the carcasses from the clean zone ($n=20$) of the slaughter line were collected. The subsequent day, samples were collected from the same 20 carcasses when leaving the cooling room to enter the cutting plant for immediate processing, as well as from two specific products obtained from these carcasses some minutes after processing, sirloin and loin. The meat products obtained from zip tie marked carcasses were monitored, collected, and labelled by staff of the industry's quality department for swab sampling, packing, cold storage, and a final swab sampling at the end of shelf-life. Additionally, surface samples were collected from the walls and floors of the cooling room ($n=8$), and knives, floors, drains, trays, and conveyors from the cutting plant ($n=13$). Loin and sirloin samples were taken at the beginning and the end of their shelf-life, packaged in a protective atmosphere with 70% oxygen and 30% carbon dioxide. The end of the commercial shelf-life for both fresh meats was determined by the manufacturer, being 7 days for loins and 9 days for sirloins, both stored at 4 °C.

In total, 157 carcass or meat cut samples and 53 surface samples (from both food contact and non-food contact environments) were collected. For a detailed list of facility surface samples and their respective sampling locations, see Table S1.

Sampling procedure

Disposable protective clothing, including gloves, footwear, and hairnets, was worn during sampling to prevent cross-contamination between samples. Gloves were changed after each sample collection. Samples were taken using Whirl–Pak Hydrated PolyProbe swabs (Whirl–Pak, WI, USA). Each sample was constituted by pooling five swabs in the same bag. On surfaces with enough space, such as floors, walls, and carcasses, an area of approximately 1 m^2 was sampled by swabbing horizontally, vertically, and diagonally, with the swab rotating in between. For carcasses, those sampled after stunning (designated as "carcass start") and after de-hairing ("carcass intermediate") were obtained by swabbing the outside (skin) of the carcass. On the other hand, those sampled at the end of the slaughter line (carcass final) and after leaving the cooling room (carcass cooling) were obtained by swabbing both external and internal half-carcasses (three external swabs and two internal swabs were pooled). For surfaces such as drains and knives, and meat cuts where swabbing 1 m^2 was not feasible, individual units were swabbed before pooling five swabs.

Once collected, the sampling bags were stored in a cooling box with ice packs. The samples taken on the first day were stored under these conditions in a cold room at the sampled industry for 24 h. At the end of the second sampling day, all samples, stored in a cooling box with ice packs, were transported to the laboratory, a journey that took less than 6 h. The day after arrival at the lab, the samples were processed by adding 10 mL of sterile phosphate buffered saline (PBS, Sigma-Aldrich, MO, USA) to each pool of five swabs and homogenising for 2 min at 175 rpm using a Stomacher (IUL Instruments, Barcelona, Spain). Subsequently, 10 mL of the homogenate was transferred to a sterile Falcon tube, followed by centrifugation at 5000×*g* for 5 min at room temperature. The supernatant was removed, and the resulting pellet was stored at − 80 °C until subsequent DNA extraction.

DNA extraction and sequencing

The DNA extraction was performed using the DNeasy PowerSoil Pro kit (Qiagen GmbH, Germany) with the modifications developed in a recent study conducted by our research group [29]. These modifications included: (i) the use of QIAamp UCP MinElute spin columns (QIAGEN) instead of standard spin columns, (ii) the addition of 600 µL of isopropanol plus 600 µL of Solution CD3 during the DNA binding step, (iii) the use of a customised wash buffer CD5, obtained by mixing CD5 and EtOH 100%, and (iv) the elution in a final volume of 40 µL by twice successive elutions of 20 µL. DNA yields were measured using a Qubit fluorometer with the dsDNA HS assay kit (Invitrogen, Thermo Fisher Scientific, USA). The obtained DNA concentrations are shown on Fig. S1.

The libraries for Illumina NovaSeq metagenomic sequencing were prepared with the Nextera DNA Flex Library Prep kit (Illumina, CA, USA) following the manufacturer's instructions. Metagenomic libraries were multiplexed using dual indexing and sequenced for 150 bp paired-end reads (average of 7.5 GB/sample) on a NovaSeq 6000 Sequencing System (Macrogen, Seoul, South Korea). Negative controls were dispatched to the sequencing company, including a total of seven negative

controls: three air samples collected from different areas of the industrial facility (slaughterline, cooling room, and cutting plant), and four empty swabs; however, due to the insufficient quantity of DNA (below the limit of detection by Qubit), they could not be subjected to sequencing.

Processing of raw reads and taxonomic annotation of reads

Adapter removal and quality trimming of raw reads was performed using TrimGalore v 0.6.0 with *–paired –nextera –stringency 5 –length 75 –quality 20 –max n 2 –trim-n* parameters (https://github.com/FelixKrueger/TrimGalore). Host DNA was removed using Bowtie2 v2.3.4.3 [30] with *–sensitive-local parameter*, removing reads from phage phiX174 (GCF 000819615), human GRCh38 (GCF 000001405.39), and *Sus scrofa* (GCF 000003025) reference genomes. The filtered reads were taxonomically assigned using Kraken2 [31] with a modified version of the PlusPF database, updated on the 12th of January 2024 (available at https://benlangmead.github.io/aws-indexes/k2), using default parameters. The modifications on PlusPF consisted of the addition of representative genomes for those species with at least one reconstructed metagenome-assembled genome (MAG) that were not included in the PlusPF database. For those species with no representative genome in public repositories, or classified as *Genus_name* sp. N, where N is an alphanumeric code, a reconstructed MAG employed for taxonomic assignment by GTDB-tk was used. Selected genomes (Table S2) were downloaded from the National Center for Biotechnology Information (NCBI) using the *FTP Path* column in the *prokaryotes.txt* file from the NCBI ftp repository (https://ftp.ncbi.nlm.nih.gov/genomes/GENOME_REPORTS/prokaryotes.txt) and downloading the*_genomic.fna.gz* file, which is the genome in.fasta format. Genome downloading was done manually on the NCBI web (https://www.ncbi.nlm.nih.gov/) for those genomes which missed the *FTP path* on the *prokaryotes.txt* file (Table S2). Downloaded genomes were decompressed and added to the kraken2 database following the instructions available at https://github.com/DerrickWood/kraken2/blob/master/docs/MANUAL.markdown. The taxid code from NCBI was added to each header of the genome (indicated on the kraken2:taxid column at Table S2) before the genome addition to the kraken2 database. Furthermore, the *Sus scrofa* reference genome employed for host removal by Bowtie was also added to the database as an extra safety check to remove host reads. Finally, Bracken [32] was employed to improve species abundance estimations, transforming the kraken2 database to Bracken format indicating a 150-bp length (*bracken-build -d PlusPF_modified/-t 64 -l 150*), which was also indicated during the Bracken run (*-r 150*).

Source tracking analysis

SourceTracker2 software [33] was used with the *–per sink feature assignments* parameter to estimate the primary sources of microbial taxa in the carcasses and meat cuts, using matrices of genera relative abundance obtained by Bracken [32]. The dataset on the contribution of source-to-sink genera was reorganised using in-house scripts (https://github.com/JoseCoboDiaz/piecharts_tax_st2). A pie chart plot was created using ggplot2 (https://github.com/tidyverse/ggplot2) to display the impact of each source on each sink.

Resistome analysis at read level

Filtered reads were aligned against the ResFinder database [34] downloaded on 24th October 2022, using bowtie2 [30] with *–very-sensitive –end-to-end* parameters. Obtained.sam files were filtered by an in-house ruby script (https://github.com/SegataLab/MASTER-WP5-pipelines/blob/master/07-AMR_virulence_genes/count_reads.rb), which removes the gene overestimation occurring when forward and reverse reads align within the same gene. The counts matrix obtained was processed by R scripts to calculate the counts per million reads (CPM) in relation to the number of reads per sample that have been taxonomically assigned to bacteria by Kraken2 [31]. Additionally, ARGs conferring resistance to critically important antibiotics were selected according to previous publications [6, 35, 36].

Resistome analysis in contigs

With the filtered reads, a de novo metagenomic assembly was performed through metaSPAdes v3.13 [37] using default parameters for each of the samples. Contigs longer than 1000 bp were taxonomically assigned using SqueezeMeta software [38], which uses Diamond [39] search against the GenBank nr database together with a rapid last common ancestor (LCA) algorithm for taxonomic assignment. Hits passing a minimum amino acid identity level of 85% and 60% for species and genus ranks, respectively, were employed for further analysis.

Contigs were aligned by BLASTn [40] against the same ResFinder database [34] version used for reads level analyses to annotate ARGs, using an 80% cut-off value for both percentage of identity and coverage. Only ARG-carrying contigs classified at the genus level were considered for further downstream analysis. ARG-carrying contigs were assigned as plasmidic or chromosomic using Platon v1.6 [41].

Binning and reconstruction of metagenome-assembled genomes (MAGs)

Reads from each sample were mapped to the corresponding sample contigs using bowtie2 v.2.2.9 [30], with parameters *–very-sensitive-local* and *–no-unal*. The *jgi_summarize_bam_contig_depths.pl* script, from MetaBAT v2.12.1 [42], was used to calculate contig depth values from the.sam files obtained by bowtie2 alignment. Contig binning was done with contigs longer than 1,500 bp using MetaBAT2, with parameters *-s 50,000 -m 1500*. Quality control of the MAGs was performed using CheckM2 v1.0.2 [43] with default parameters. Only high quality (completeness > 90%, contamination < 5%) and medium quality (completeness between 50 and 90%, contamination < 5%) MAGs were kept for further analysis, according to parameters proposed by [44]. Taxonomic assignment of MAGs was performed by GTDB-Tk v2.4.0 [45] using the *classify_wf* command and release 220 of the database. Additionally, those MAGs belonging to more prevalent species were employed for the calculation of Average Nucleotide Identity (ANI) values between MAGs from the same species using dRep v2.6.2 [46]. For each analyzed species, the "ani" column from the N*db.txt* output file obtained from dRep was transformed into a distance matrix, which was used to construct a phylogenetic tree plot by *ggtree* R-package, using the UPGMA clustering algorithm (*hclust* method = "average").

Finally, MAGs assigned to genus and/or species with no Latin name (i.e. Phil1 sp004558525) were considered as "uncultured genus" and "uncultured species" to estimate the number of uncultured bacteria found by this metagenomic approach.

Statistical analysis

Alpha-diversity was analysed by calculating richness and Simpson's diversity index for taxonomy at the species level and resistome at the gene level, using the *vegan* R-package (https://github.com/vegandevs/vegan). Data distribution and homoscedasticity were tested by the Shapiro test and Levene test, using the *car* R-package. Due to the results shown by these two tests, a nonparametric Wilcoxon test was chosen to perform the comparisons through the *ggpubr* R-package (https://github.com/kassambara/ggpubr). The beta-diversity was estimated by principal coordinate analysis using Bray–Curtis dissimilarities and the *vegdist* function, while within-group dispersion was assessed using the *betadisper* function, being both functions in the *vegan* R-package. Finally, the *adonis* function in the *vegan* R-package was used to check for within-group differences. The compare means function in the *ggpubr* R-package was used to include statistically significant differences in the boxplot figures, which were plotted using the *ggplot2* R-package.

Comparisons between samples of multiple groups for the abundance of taxa and ARGs, alpha diversity indices, and total amount of ARGs were performed using the Kruskal–Wallis test and the Wilcoxon signed-rank post hoc test. *p* values were adjusted by Benjamini and Hochberg's method, and significance was set at $p < 0.05$.

Results

Taxonomic diversity along the processing line

The taxonomic profile of the microbiome of carcasses and meat cuts was significantly influenced by the sampling point (adonis: $R^2 = 0.534$, $p = 0.001$), while farm of origin and sampling time (beginning vs. end of the morning working day) did not have a significant effect (Fig. 2A; Table S3). However, these two variables exerted a notable influence on specific points of the processing line, particularly on the early stages of carcass processing at the slaughterhouse (carcass start and carcass intermediate) (Table S3 and Fig. S2). Beta-dispersion was significantly lower at carcass start and intermediate than at the rest of the sampling points ($p < 0.001$) (Fig. S3A).

Species richness was significantly higher on carcasses at the initial and intermediate stages of processing ($p < 0.0001$) than on those at the end of the slaughter line or leaving the cooling room. Moreover, carcasses sampled at the end of the cooling process exhibited higher Simpson index values than those in earlier stages of processing ($p < 0.0001$) (Fig. 2B). Regarding the meat cuts, there were no significant differences in alpha-diversity values between the loin and sirloin at the beginning of the product's shelf-life. However, both products suffered a significant decrease in the Simpson index along their shelf-life ($p < 0.0001$) (Fig. 2B).

The most abundant taxa identified on carcasses varied according to the point in the processing line (Fig. 2C and Fig. S3A). At the initial sampling point of the carcasses (carcass start), the most predominant genera were *Streptococcus* (16.9%) and *Clostridium* (9.7%) (Fig. 2C), and the most abundant species were *Streptococcus alactolyticus* (14.6%) and *Clostridium* sp. CAG:221 (5.5%) (Fig. S3A). However, in subsequent carcass sampling points (carcass intermediate, carcass final, and carcass cooling), a notable shift was observed in the taxonomic profile, with the emergence of genera such as *Acinetobacter*, *Anoxybacillus*, *Microbacterium, Serratia*, *and Pseudomonas* (Fig. 2C), with the most abundant species being *Acinetobacter johnsonii, Anoxybacillus flavithermus*, *Microbacterium* sp. PAMC22086, *Serratia liquefaciens*, and *Pseudomonas benzenivorans* (Fig. S3C). Moreover, a further noteworthy alteration in the relative abundance of bacterial taxa was identified in the meat cuts. At the

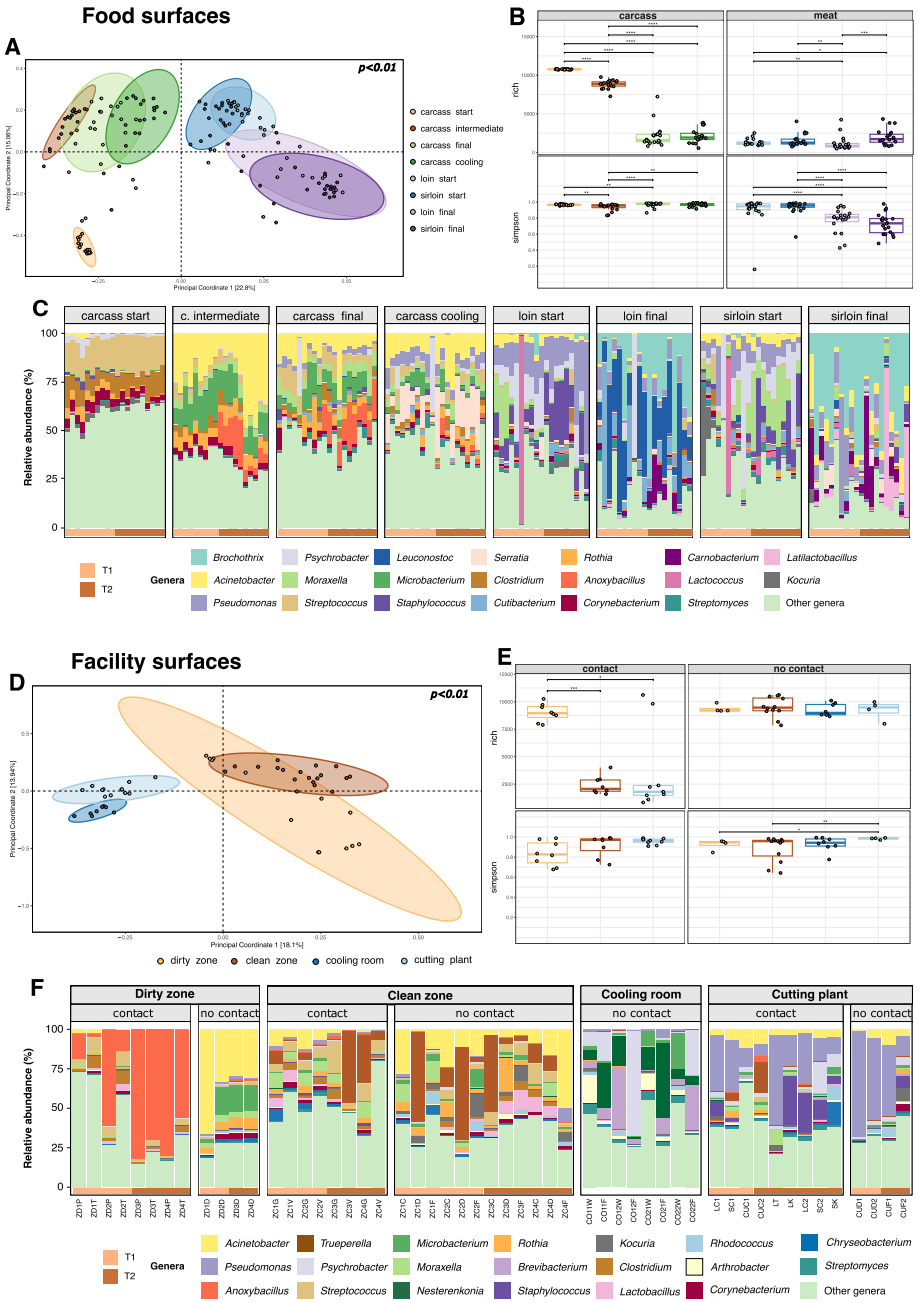

Fig. 2 Changes in alpha diversity, beta diversity and bacterial taxonomy on carcasses, meat cuts and surfaces along the processing line. Microbiome analysis on carcasses and meat cuts ($n = 157$): **A** Principal Coordinates Analysis (PCoA), using Bray–Curtis distance (Adonis test values are provided in Table S3); **B** Richness and Simpson indices at the species level, where only significant p-values ($p < 0.05$) from the Wilcoxon signed-rank test analysis are indicated (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ and ****: $p < 0.0001$); and **C** relative abundance of the 20 most dominant bacterial genera, other bacterial genera are grouped into the category "Other genera" and statistical test values are provided in Supplementary file 1. Microbiome analysis on contact and non-contact surfaces ($n = 53$): **D** Principal Coordinates Analysis (PCoA), using Bray–Curtis distance (Adonis test values are provided in Table S3); **E** Richness and Simpson indices at the species level, where only significant p-values ($p < 0.05$) from the Wilcoxon signed-rank test analysis are indicated (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ and ****: $p < 0.0001$); and **F** relative abundance of the 20 most dominant bacterial genera, other bacterial genera are grouped into the category "Other genera" and statistical test values are provided in Supplementary file 1

beginning of the shelf-life, the dominant genera in loin and sirloin were *Pseudomonas* (17.9% and 8.7%, respectively), *Staphylococcus* (13.8% and 8.4%), *Psychrobacter* (11.1% and 14.3%), and *Moraxella* (5.4% and 12.6%) (Fig. 2C). On the other hand, at the end of the meat cuts' shelf-life, the most abundant genera in loin were *Leuconostoc* (27.9%), *Brochothrix* (23.7%), and *Cutibacterium* (10.6%), while those in sirloin were *Brochothrix* (39.3%), *Pseudomonas* (10.1%), and *Carnobacterium* (8.5%) (Fig. 2C). The main changes observed throughout the shelf-life of the meat cuts were the significant increase in the relative abundance of the genus *Brochothrix*, particularly the species *Brochothrix thermosphacta* in both loin (23.8%) and sirloin (39.4%) ($p < 0.0001$). Additionally, there was a notable increase in the relative abundance of *Leuconostoc* and *Cutibacterium* in loin, with *Leuconostoc carnosum* (19.4%) and *Cutibacterium acnes* (10.6%) being the most abundant species ($p < 0.05$), and of *Carnobacterium* and *Latilactobacillus* in sirloin, with a particularly pronounced increase of *Latilactobacillus sakei* (8.0%) ($p < 0.0001$) (Fig. 2C, Fig. S3A, and Supplementary file 1). The most notable differences between the two products were observed towards the end of their shelf-life. The genus *Brochothrix,* especially the species *B. thermosphacta,* showed a significantly higher relative abundance in sirloin than in loin ($p < 0.05$), while *Leuconostoc* and *Cutibacterium,* particularly *L. carnosum* and *C. acnes,* were more dominant in loin than in sirloin ($p < 0.001$) (Fig. 2C, Fig. S3A, and Supplementary file 1).

Remarkably, some of the most abundant taxa in carcasses and meat cuts demonstrated a notable increase in abundance towards the end of the morning workday (sampling time T2, as compared to T1). This was the case of *Anoxybacillus* in carcass intermediate ($p < 0.001$); *Staphylococcus* in loin and sirloin at the beginning of their shelf-life ($p < 0.01$); *Cutibacterium* in loin at the beginning of its shelf-life ($p < 0.05$); *Moraxella* in sirloin at the beginning of its shelf-life ($p < 0.05$); and *Brochothrix* ($p < 0.05$) and *Carnobacterium* at the end of loin's shelf-life ($p < 0.01$) (Fig. 2C and Supplementary file 1).

Regarding food processing environments, the taxonomic profile of their microbiome was significantly influenced by the variables processing area (slaughter line dirty zone, slaughter line clean zone, cooling room, cutting plant) (adonis: $R^2 = 0.330$, $p = 0.001$) and surface type (food contact vs non-contact) (adonis: $R^2 = 0.09$, $p = 0.001$) (Fig. 2D; Table S3). Ordination analyses revealed clear differences in taxonomic profile among samples from different processing areas, which grouped separately in the principal coordinate analysis, with higher beta-dispersion in the slaughter line areas compared to the cooling room and cutting plant processing environments (Fig. 2D). Beta-dispersion revealed no statistically significant disparities among the different processing areas sampled (Fig. S3C).

In terms of alpha diversity, richness values were significantly higher on contact surfaces from the slaughter line dirty zone than on contact surfaces from the slaughter line clean zone ($p < 0.05$) and the cutting plant ($p < 0.001$), while differences observed among surface types in Simpson's index values were less pronounced (Fig. 2E).

The most abundant taxa in the processing environments changed depending on the processing area being sampled (Fig. 2F and Fig. S3B). In the slaughter line surfaces, *Anoxybacillus*, *Acinetobacter and Trueperella* were the most abundant genera, and *A. flavithermus, A. johnsonii* and *Trueperella pyogenes* were the most abundant species. *Nesterenkonia* (16.2%), *Psychrobacter* (15.9%) and *Brevibacterium* (12.2%) were the main genera in the surfaces of the cooling room and *Nesterenkonia sandarakina* and *Psychrobacter* sp. P11F6 were the main species, while in the cutting plant *Pseudomonas*, *Acinetobacter* and *Staphylococcus*, and the species *T. pyogenes, Pseudomonas lundensis* and *Staphylococcus aureus* were the most abundant ones (Fig. 2F and S2B and Supplementary file 1). Remarkably, some of the taxa frequently found in high abundance on carcasses and meat cuts were also identified with high relative abundance on various processing surfaces (Fig. 2F). For example, the genus *Anoxybacillus,* and particularly *A. flavithermus*, was present with high relative abundances on contact surfaces from the dirty zone of the slaughter line (28.5%), as well as on carcasses at intermediate (3.5%) and final stages of the slaughter line (4.4%). Likewise, *Acinetobacter,* and especially *A. johnsonii,* was notably abundant on non-contact surfaces from the dirty and clean zones of the slaughter line, with an abundance of 22.8% and 10.3%, respectively, and was also frequently identified with high relative abundances on carcasses at intermediate (18.5%) and final stages of the slaughter line (4.4%) and leaving the cooling room (5.1%) (Fig. 2F and Fig. S3B). In the cutting plant, a similar observation was made for the genera *Pseudomonas*, particularly *P. lundensis,* and *Staphylococcus*, identified on meat cuts and on processing surfaces in contact with loin (36.2% and 16.6%, respectively) and sirloin (19.4% and 6.4%, respectively) (Fig. 2F).

Tracking the source of bacterial taxa contaminating carcasses and meat cuts

Source tracking analyses were conducted to assess the influence of various facility surfaces and processing steps on the bacterial taxonomic profile of carcasses and meat cuts. The results revealed that the bacterial composition of the microbiome of carcasses at intermediate and final stages of the slaughter line, and those leaving the cooling room, was highly impacted by the microbiome of

processing surfaces from the slaughterhouse, while the microbiome of carcasses at the start of the slaughter line exhibited a relatively lower influence. The carcasses at the start of the slaughter line represented the main source of the species *S. alactolyticus*, *Clostridium sp.* CAG:221, *Limosilactobacillus reuteri*, and *Rothia nasimurium*, among other minority taxa (Fig. 3A). Surfaces in the dirty zone and clean zone of the slaughter line were an important source of abundant taxa on the carcasses, such as *A. flavithermus, A. johnsonii, M. osloensis*, or

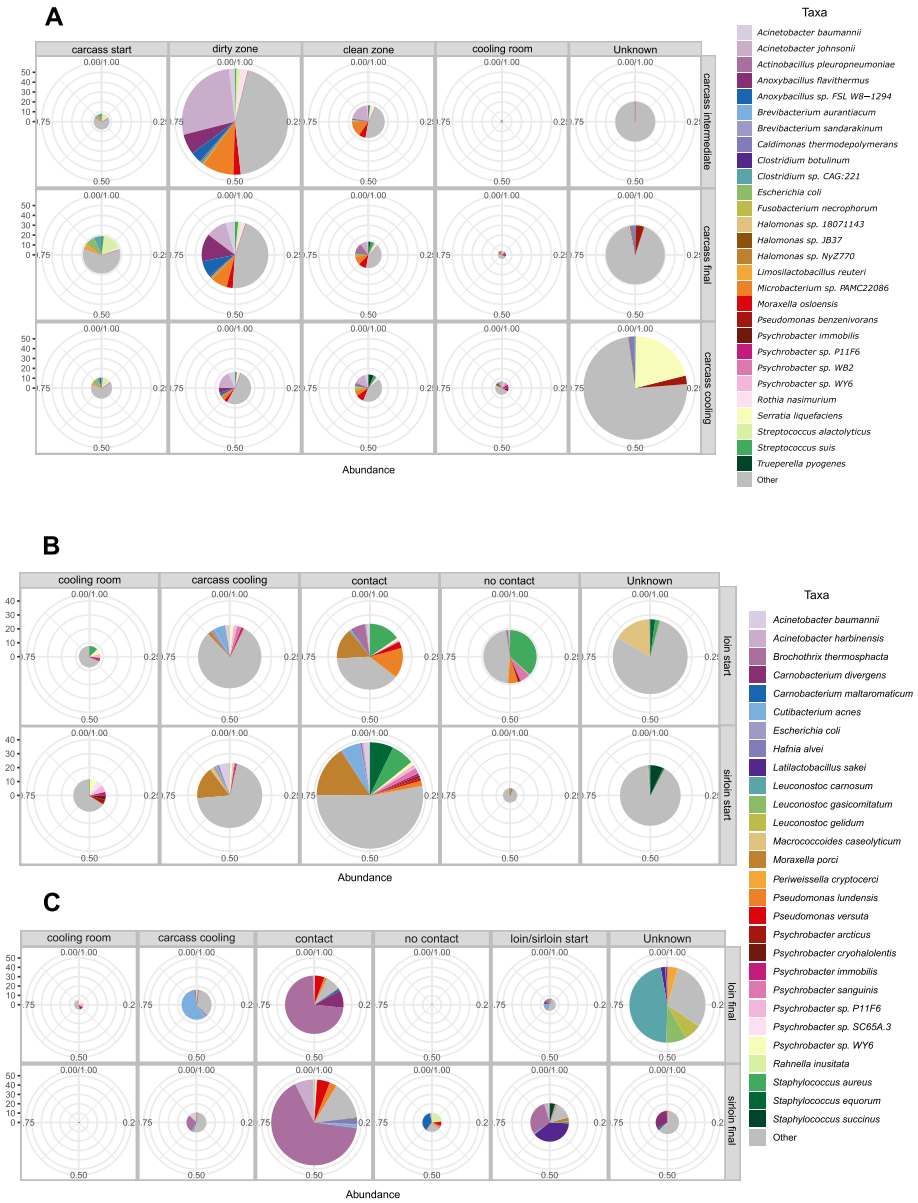

Fig. 3 Bacterial source attribution of carcasses and meat samples calculated by SourceTracker2 software. Pie chart plots represent bacterial sources (columns, source samples) for the carcasses and meat cuts (rows, sink samples). The size of each pie chart is proportional to the percentage of source sample influence on sink sample (indicated on the y-axis). The colors within each pie chart indicate the percentage of species influence for each source-sink pair. **A** Taxonomic attribution of carcasses at the start of the slaughter line and slaughter line and cooling room surfaces as a source of bacteria for carcasses at intermediate and final stages of the slaughter line and carcasses leaving the cooling room. The 28 main species with significant influence were represented, while other species were grouped in the category "Other". **B** Taxonomic attribution of carcasses leaving the cooling room, and surfaces of the cooling room and cutting plant as sources of bacteria for loin and sirloin at the beginning and end of their shelf-life. The 28 main species with significant influence shared at the beginning and the end of the products' shelf-life were represented, while other species were grouped in the category "Other"

Microbacterium sp. PAMC22086 (Fig. 3A). However, the influence of the dirty zone surfaces decreased along the processing line and was lower on the carcasses leaving the cooling room (Fig. S4A). The surfaces of the cooling room were identified as a source of *Psychrobacter* spp. on the carcasses after cooling. Nonetheless, the impact of the cooling room surfaces on the microbiome of carcasses was lower compared to that of the microbiome of the surfaces from the dirty and clean zones of the slaughter line (Fig. 3A). Moreover, the proportion of genera with an unknown origin increased along the processing line. Among them, some were particularly abundant in the carcasses, such as *S. liquefaciens* and *P. benzenivorans* (Fig. 3A).

The carcasses leaving the cooling room and the contact surfaces from the cutting plant were identified as the sample categories having the strongest impact on the composition of the microbiome of loin and sirloin at the beginning of their shelf-life. They were identified as the primary source of species such as *P. lundensis, S. aureus, Psychrobacter* spp., *M. porci*, *and C. acnes*. The main species identified were *P. lundensis*, *Pseudomonas vertusa*, and *S. aureus* in contact surfaces, and *M. porci, C. acnes*, and *S. aureus* in contact surfaces and carcasses after cooling. Moreover, the cooling room surfaces represented a source of *Psychrobacter* spp. despite their minimal impact on meat cuts (Fig. 3B).

For the meat cuts at the end of the shelf-life, the impact of the microbiome of the carcasses after cooling was low, while the influence of contact and non-contact surfaces from the cutting plant was notably higher (Fig. 3C). *B. thermosphacta*, one of the most abundant species in loin and sirloin, and others less prevalent such as *P. lundensis* and *P. versuta*, were traced back to contact surfaces in the cutting plant, while *C. acnes* was traced back to carcasses after cooling in the case of loin. The proportion of taxa with an unknown origin was considerable, including abundant species such as *L. carnosum* in loin and *Carnobacterium divergens* in sirloin (Fig. 3C).

Reconstruction of metagenome-assembled genomes (MAGs)

A total of 1,291 medium or high-quality MAGs were reconstructed, comprising 325 high quality MAGs (224 from carcass, 94 from environmental, and 7 from meat samples) and 966 medium quality MAGs (785 from carcass, 171 from environmental, and 10 from meat samples) (Table S4). The average number of MAGs obtained by sample was 43.8 for carcass start, 8 for drains, 7.4 for non-contact surfaces, 4.9 for contact surfaces, 4.7 for carcass intermediate, and 1.7 for loin-sirloin start. The MAGs were classified into 183 genera (including 32 uncultured and 6 unknown genera) and 270 species (including 97 uncultured and 50 unknown species), with 109 MAGs classified as unknown species (Fig. 4A). Carcass start samples harbor 36 and 82 unculturable genera and species, respectively. The species with the most number of MAGs reconstructed were *Streptococcus faecavium* (42 MAGs), *Rothia endophytica* (33 MAGs), *Microbacterium maritypicum* (27 MAGs), *Acinetobacer johnsonii* (27 MAGs), *Limosilactobacillus reuteri* (22 MAGs), together with other uncultured species and genera (Fig. 4B). Regarding the distribution of MAG species among samples, the most abundant species were found mainly in carcass start samples, mainly due to the amount of MAGs recovered from these samples, but MAGs from some species such as *A. flavithermus*, *A. johnsonii*, and *M. maritypicum* were mainly found in carcass intermediate and environmental samples (Fig. 4B).

The previously indicated most abundant species, together with *Streptococcus hyointestinalis*, *Anoxybacillus flavithermus*, and *Suicoccus acidiformans* (18 MAGs for each species), were employed for strain analysis by ANI calculation (Fig. 4C). Some of these species, such as *A. flavithermus*, *A. johnsonii*, *R. endophytica*, *S. acidiformans*, and *S. hyointestinalis*, were mainly represented by a different strain per sample, with clear clustering of strains by zone (carcass-dirty zone vs. clean zone) for *A. johnsonii*, by surface (carcass start vs. drains-carcass intermediate) for *R. endophytica*, and by time (T1:G1-G2 vs. T2:G3-G4) for *S. intestinalis* (Fig. 4C). On the other side, *L. reuteri* had a clear cluster of 17 MAGs from carcass start belonging to the same strain, which separated from MAGs from environmental samples; *M. maritypicum* MAGs from carcass and dirty zone (almost all of them from the same strain) were clearly separated from MAGs from the clean zone; and the majority of *S. faecavium* MAGs belonged to the same strain (Fig. 4C).

The abundance and diversity of ARGs along the processing line

The analysis of the resistome revealed a decrease ($p < 0.0001$) in the counts of ARGs per million reads (CPM) from the initial carcasses to the rest of the carcasses sampled and meat cuts. Indeed, the lowest ARGs CPM were found in the meat cuts at the start of their shelf-life. Nevertheless, carcasses sampled in the slaughter line revealed a significant increase in the ARGs CPM from carcass intermediate (after de-hairing) to carcass final (after evisceration and before cooling). In the analysis of meat cuts, an increase in the amount of ARGs was found during the storage of the products under foreseeable conditions for commercialisation, especially in the case of sirloin (Fig. 5A).

Beta-diversity analyses of the resistome revealed an increase in beta-dispersion along the processing line

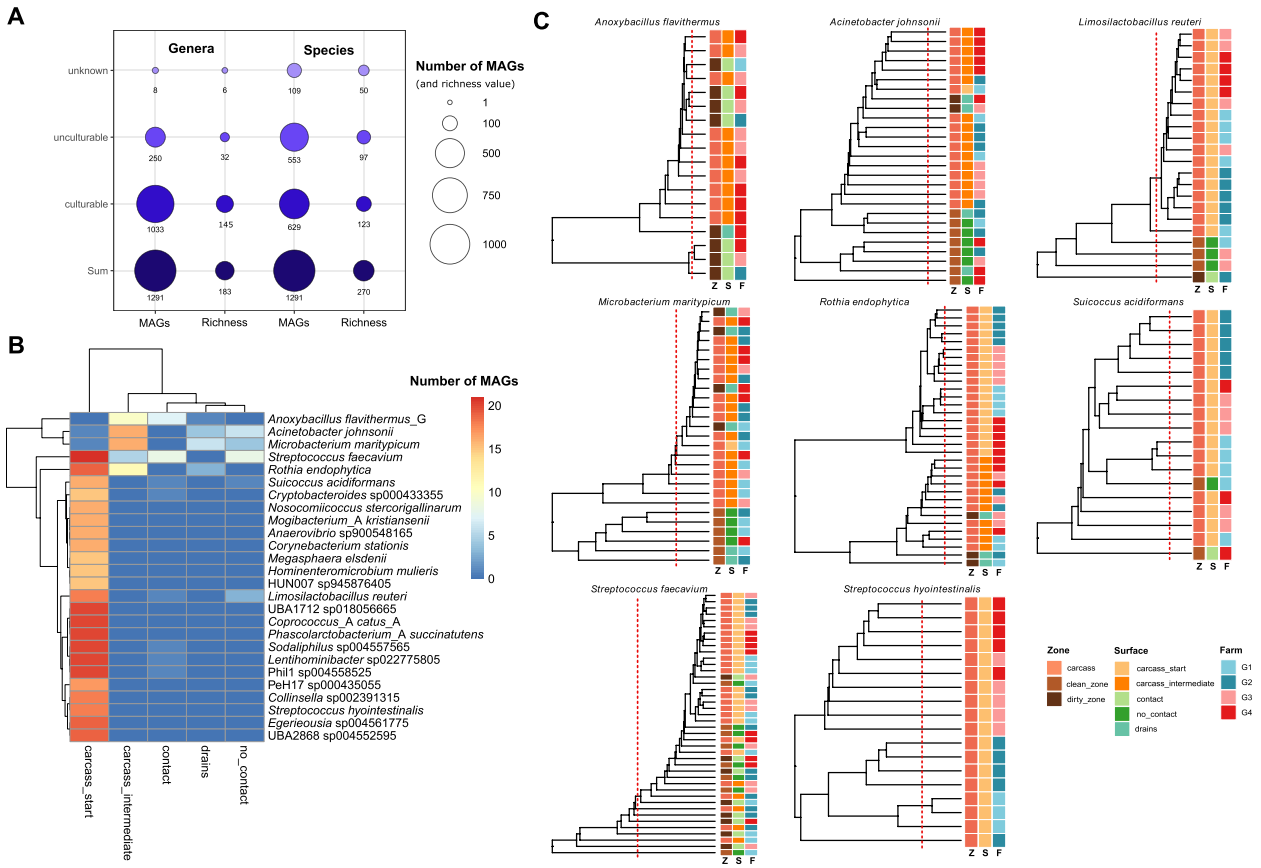

Fig. 4 MAGs obtained through per-sample assembly and binning of metagenomic reads. **A** Number of MAGs classified as unknown and uncultured genera and species ("MAGs" columns) and total number of unknown and uncultured genera and species obtained ("Richness" columns). **B** Heatmap of number of MAGs from the most represented species and their distribution on carcasses and environmental samples. **C** Phylogenetic tree constructed using the ANI distance matrix generated by the dRep software with the most important species according to the number of MAGs and their distribution among different surfaces. Dashed red line indicates the limit to consider same strain (ANI value > 99.9%)

for both carcasses and meat cut samples (Fig. 5B). The variable sampling point explained 32.3% of the variation (adonis: $R^2 = 0.323$, $p = 0.001$). On the other hand, the variables farm of origin and sampling time (beginning and end of the morning working day) did not significantly influence ordination (Table S3). However, as with taxonomy, these two variables exerted a notable influence on specific points of the processing line, particularly on the early stages of carcass processing at the slaughterhouse (carcass start and carcass intermediate) (Fig. S5 and Table S3). Beta-dispersion was significantly lower at carcass start and intermediate and sirloin at the end of its shelf-life than at the rest of the sampling points ($p < 0.001$) (Fig. S3B). The carcass start was the sampling point with the lowest beta-dispersion.

A significant decrease ($p < 0.001$) was observed in the richness and diversity of ARGs along the production line for carcasses (Fig. 5C), while for the meat cuts, a reduction in the Simpson's index ($p < 0.001$) was found along shelf life for sirloin. Moreover, richness was significantly higher in sirloin than in loin at the end of their shelf life ($p < 0.001$).

The most abundant ARGs in carcasses and meat cuts were associated with resistance against tetracyclines (65%), aminoglycosides (8%), and lincosamides (7.3%) (Fig. 5D and Supplementary file 2). A wide diversity of different ARGs from each of the main antimicrobial families was found in the early stages of carcass processing at the slaughterhouse (especially for carcass start). Thus, for instance, many *tet* genes were detected, with *tet(L)* (24%), *tet(W)* (13%), and *tet(W/32/O)* (8%) being the most abundant genes associated with tetracycline resistance (Fig. 5E). However, in later stages of carcass and meat processing, just a few ARGs remained. For instance, *tet(L)*, which became highly abundant and predominant in sirloin at the end of its shelf-life, showed a significant increase compared to the beginning of shelf-life ($p < 0.0001$) (see in Supplementary file 2). Remarkably,

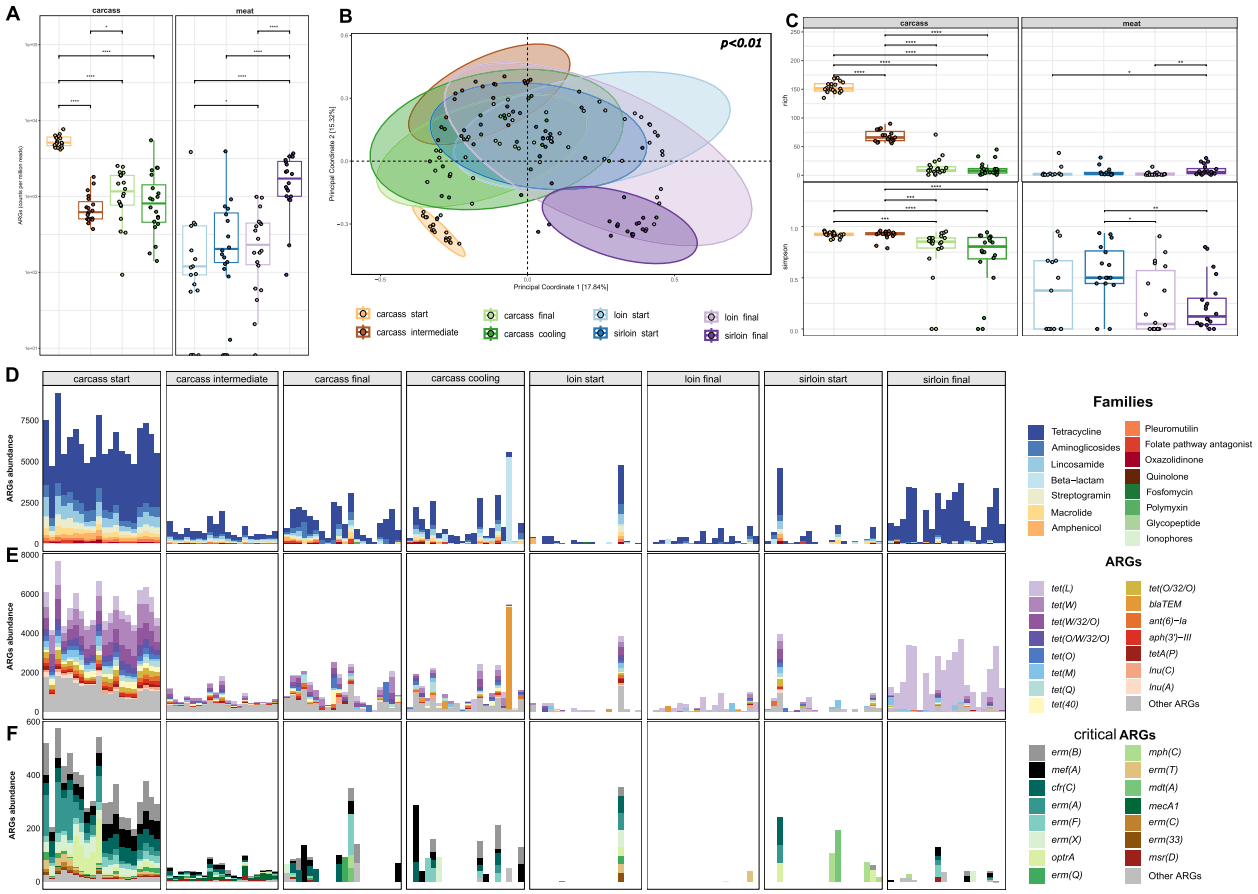

Fig. 5 Changes in the resistome along the production line in carcasses and meat cuts. **A** ARGs abundance in carcasses and meat cut samples, inCPM, with significant p values ($p < 0.05$) from the Wilcoxon signed-rank test analysis indicated. **B** PCoA, using Bray–Curtis distance, at the ARGs level for carcasses and meat cuts ($n = 157$). Adonis test values are indicated in Table S3. **C** Richness and Simpson's indices calculated with the ARG–CPM matrix, with only significant p values ($p < 0.05$) from the Wilcoxon signed-rank test analysis being indicated. Barplots showing the 15 most abundant ARGs classes (the multi-drug resistant ARGs were allocated to each of the associated families) (**D**), ARGs (**E**), and ARGs associated with resistance to antibiotics of critical importance (**F**). 'Other ARGs' refers to the remaining ARGs genes or classes grouped together. Statistical test values are provided in Supplementary file 2

various ARGs of critical importance considering the resistance phenotypes they are associated with were identified in our analyses, with *erm(B)* (16%), *mef(A)* (16%), and *cfr(C)* (14%) being the most abundant (Fig. 5F). The majority of the most abundant critical ARGs found belonged to the *erm* family, associated with resistance to macrolides, lincosamides, and streptogramins. However, their abundance and diversity also decreased along processing, and only a few critical genes were occasionally detected on meat cuts at the end of their shelf-life.

Surfaces of the slaughter line exhibited a higher amount of ARGs (in CPM count) compared to those of the cooling room and cutting plant ($p < 0.001$), with the cooling room surfaces showing the lowest ARGs CPM detected (Fig. 6A). Within the slaughter line, the clean zone surfaces sampled had a higher ARGs CPM compared to the dirty zone surfaces sampled ($p < 0.05$). The variables processing area (slaughter line dirty zone, slaughter line clean zone, cooling room, cutting plant) and surface type (contact vs non-contact) explained, respectively, 25.7% (adonis: $R^2 = 0.257$, $p = 0.001$) and 6.2% (adonis: $R2 = 0.062$, $p = 0.001$) of the variation observed in the beta-diversity analyses (Table S3). Ordination analyses showed a separation of samples from clean and dirty zones of the slaughterhouse, the cooling room and the cutting plant based on their resistome profile (Fig. 6B). Beta-dispersion levels were found to be significantly higher at the surfaces in the cooling room and cutting plant than at the surfaces in the dirty and clean zones ($p < 0.05$) (Fig. S3D).

A significant decrease along the processing line was also observed in resistome richness and Simpson's diversity indices for contact surfaces ($p < 0.001$ and $p < 0.05$, respectively), and for non-contact surfaces from the

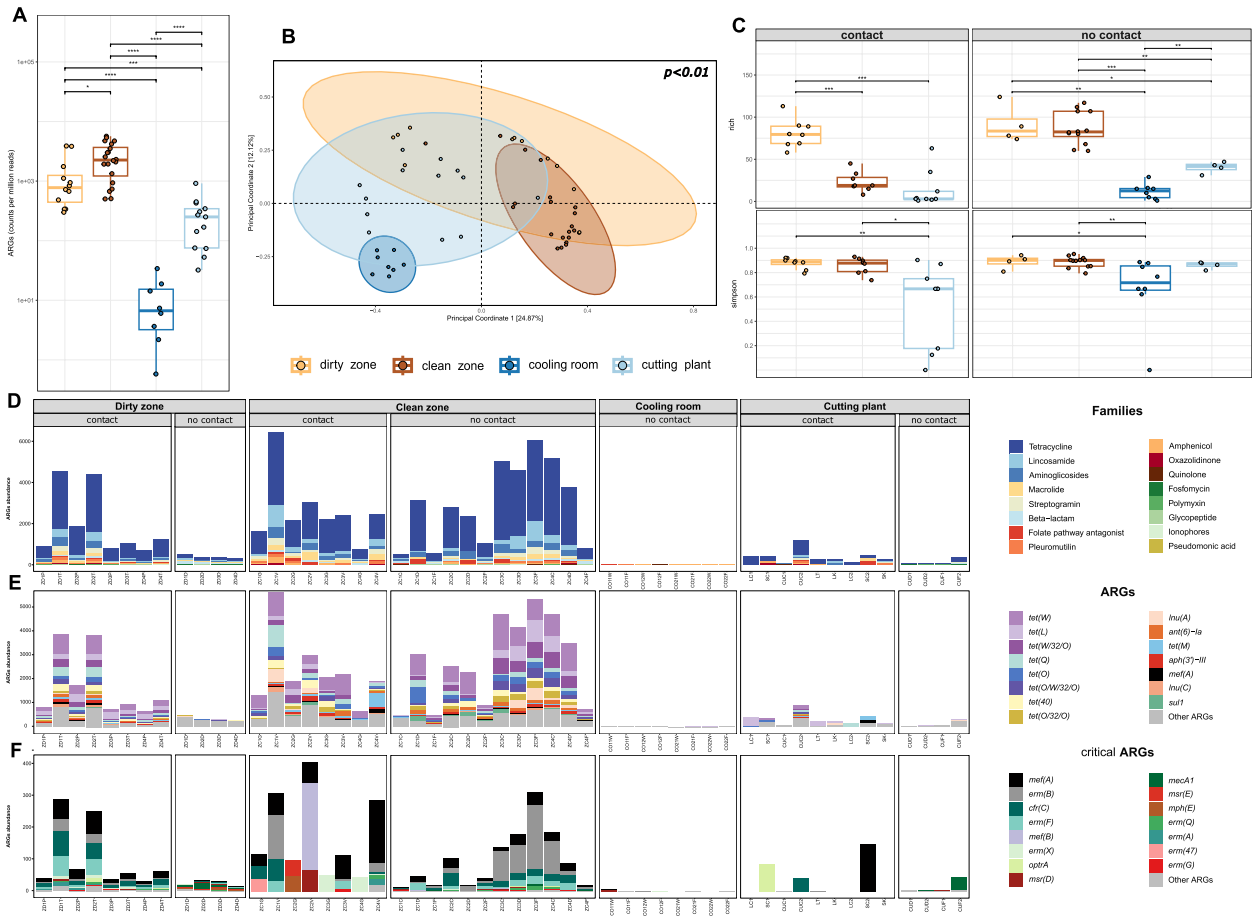

Fig. 6 Changes in the resistome of food contact and non-contact surfaces. **A** ARGs abundance in contact and non-contact surfaces, in CPM, with significant p values ($p < 0.05$) from the Wilcoxon signed-rank test analysis indicated. **B** PCoA, using Bray–Curtis distance, at the ARGs level for the dirty zone ($n = 12$), clean zone ($n = 20$), cooling room ($n = 8$) and cutting plant ($n = 13$) samples. Adonis test values are indicated in Table S3. **C** Richness and Simpson's indices calculated with the ARG–CPM matrix, with only significant p values ($p < 0.05$) from the Wilcoxon signed-rank test analysis being indicated. Bar plots showing the 15 most abundant ARGs classes (the multi-drug resistant ARGs were allocated to each of the associated families) (**D**), ARGs (**E**), and ARGs associated with resistance to antibiotics of critical importance (**F**). 'Other ARGs' refers to the remaining ARGs genes or classes grouped together. Statistical test values are provided in Supplementary file 2

slaughter line surfaces to the cooling room ($p < 0.05$), while surfaces of the cutting plant showed higher ARGs richness than surfaces of the cooling room ($p < 0.01$) (Fig. 6C).

The main ARGs found on the processing environments were again mainly associated with resistance to tetracyclines (64%), lincosamides (8.5%), and aminoglycosides (7.4%) in both contact and non-contact surfaces (Fig. 6D). The most frequently detected ARGs were *tet(W)* (20%), *tet(L)* (10%), and *tet(W/32/O)* (7.4%), all linked to tetracycline resistance and predominantly found on contact surfaces in both the dirty and clean zones of the slaughter line, as well as on non-contact surfaces of the slaughter line clean zone (Fig. 6E; Supplementary file 2). Furthermore, the most abundant ARGs of critical importance previously found on carcasses and meat cut samples were also present on the factory surfaces: *mef(A)* (30%), *emr(B)* (15.3%), and *cfr(C)* (14.6%) (Fig. 6F). The occurrence of ARGs in cutting plant surfaces was low, restricted to some particular samples where some ARGs, such as *tet(L)* and *tet(W)*, which are also commonly found in meat cuts, prevail (Fig. 6E).

Main genera associated with ARGs

An assembly-based analysis of the resistome was performed to improve the understanding of the genetic background of the ARGs identified. This approach allowed the identification of the main taxa carrying ARGs and the association of ARGs with mobile genetic elements (MGEs). Out of the total 2,112,095 contigs

obtained, 1927 (0.091% of contigs) were found to carry ARGs, and 107 ARG-carrying contigs (5.55%) were classified as plasmidic.

The main ARGs identified on carcasses and meat cuts were associated with resistance to tetracyclines, beta-lactams, aminoglycosides, and Lincosamides-Pleuromutilins-Streptogramin A (LSP), mostly assigned to the *Staphylococcus* and *Acinetobacter* genera. *Staphylococcus* exhibited resistance to a wide range of antibiotic families, while *Acinetobacter* mostly harbored ARGs for tetracyclines and beta-lactams resistance (Fig. 7A). Additionally, *Staphylococcus* was the predominant genus associated with ARGs in carcasses at the start of the slaughter line, linked to resistance to various classes of ARGs, including tetracyclines, LSP, and Macrolides-Lincosamides-Streptogramin B (MLS). Most of them were associated with plasmids, although ARGs associated with LSP resistance were mainly identified in chromosomic contigs (Fig. 7B). Some of these ARGs assigned to *Staphylococcus* were classified as of critical importance, such as *erm(C)* and *mec(A)*, while others were associated with multidrug resistance, including *vga(E)* and *vga(A) LC* (Fig. S6A). However, in carcasses at intermediate stages of the slaughter line, the majority of ARGs detected in contigs were assigned to *Acinetobacter* and were associated with resistance to tetracyclines on plasmids and beta-lactams on chromosomes (Fig. 7B). The resistance to tetracyclines observed in contigs assigned to *Acinetobacter* was mainly attributed to the *tet(39)* gene, frequently found on plasmids, while resistance to beta-lactams was mainly linked to the carriage of *blaOXA-333* (Fig. S6A, B).

ARGs detected in processing environments were mainly associated with the *Acinetobacter* genus and linked to beta-lactams (in chromosomic contigs) and

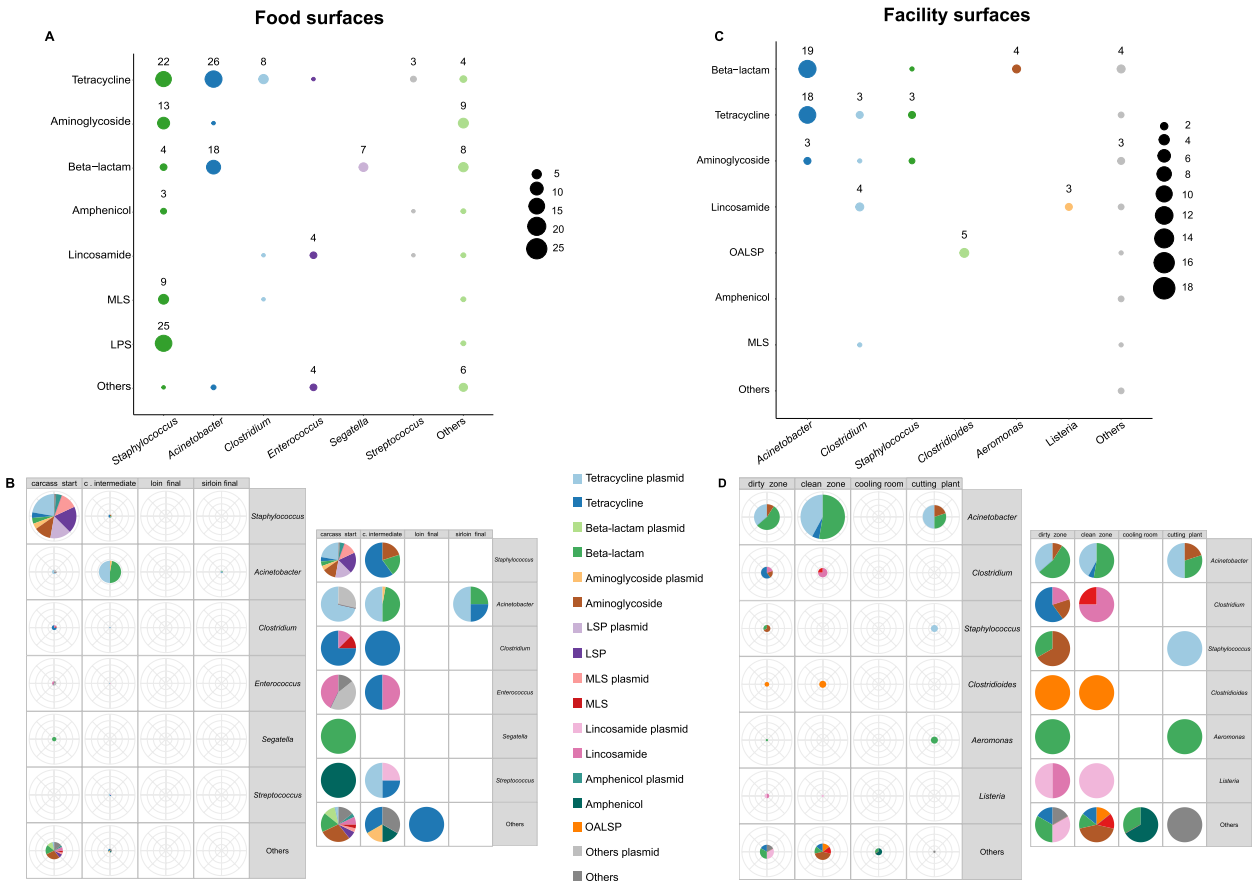

Fig. 7 Taxonomic assignment of ARG-carrying contigs. The left side of the figure illustrates food surfaces (carcass and meat cuts), while the right side of the figure details facility surfaces, such as contact and non-contact surfaces. **A** Number of contigs within the seven most abundant ARGs classes, and assigned to the six most abundant genera, detected in carcasses and meat cuts. **B** Distribution of ARGs classes by genus and sample type on carcasses and meat cuts. **C** Number of contigs within the seven most abundant ARGs classes, and assigned to the six most abundant genera, detected in contact and non-contact surfaces. **D** Distribution of ARGs classes by genus and sample type on processing environmental samples. The size of circles represents the number of contigs per genus and surface. The ARGs classes are expressed as a proportion (%) in each genus. MLS: Macrolides, Lincosamides and Streptogramin B. LSP: Lincosamides, Streptogramin A and Pleuromutilins. OALSP: Oxazolidinones, Amphenicols, Lincosamides, StreptograminA and Pleuromutilins

tetracyclines (in plasmidic contigs) resistance, predominantly found on surfaces in the dirty and clean zones of the slaughterhouse and the cutting plant (Fig. 7C, Fig. D). The main tetracycline resistance gene identified was *tet(39)*, while for beta-lactams resistance the main genes were *blaOXA-212* and *blaOXA-282* (Fig. S6C). Notably, among other minority taxa identified as carriers of ARGs in processing environments, *Listeria* was found associated with the *lnu(A)* gene, linked to lincosamides resistance and harboured on plasmidic contigs (Fig. S6D).

Of the 107 ARG-carrying contigs classified as plasmidic, seven were found to contain more than two ARGs. Five of these contigs exhibited the same ARGs associated with resistance to aminoglycosides (aph(3')–Ib and *aph(6')–Id)* and folate pathway antagonists (*sul1*) and were obtained from different samples, including non-contact surfaces from the slaughterhouse clean zone, contact surfaces from the slaughterhouse dirty zone, and carcass intermediate samples (Fig. S7).

Discussion

The current study provides a detailed description of the bacterial taxa that prevail in carcasses throughout the production line and in two different meat cuts, sirloin and loin, at both the beginning and end of their shelf-life. Most of the main taxa detected, such as *Anoxybacillus*, *Acinetobacter*, *Moraxella, Psychrobacter*, *Brochothrix*, *Pseudomonas*, *Trueperella*, and *Staphylococcus*, have been previously identified in meat processing environments and meat products [6, 16, 27, 47, 48]. Moreover, the most prevalent bacteria commonly associated with the spoilage of refrigerated beef and pork include some of the genera found with high relative abundances in this study, such as *Brochothrix* (*B. thermosphacta*), *Carnobacterium*, *Leuconostoc*, and *Pseudomonas* [2, 48].

Microbial richness decreased along the processing line, and diversity decreased during the meat cuts' shelf-life, suggesting that the processing of carcasses and meat may be an important factor shaping the product microbiome and leading to the emergence of some dominant taxa. Moreover, beta-diversity analyses indicated that the taxonomic profile was significantly influenced by the point of sampling and the processing room. These results, together with the lack of significant differences among carcass and meat cut samples from the different farms, suggest that the facility surfaces and the microbial succession dynamics induced by the conditions prevailing along processing, distribution, and shelf-life have a greater impact on the microbiome of final products than the microbiome of the animals entering the slaughterhouse. Moreover, SourceTracker analyses supported this hypothesis of deep industry environment influence on meat microbiomes.

The significant differences observed among facility rooms can be due to the impact of factors like environmental conditions, such as temperature/humidity, faecal contamination, or frequency of cleaning and disinfection, among others [4, 6, 15, 16, 49, 50]. In fact, the (co)occurrence of some microbial taxa could be related to their habitat and physiological characteristics. In the initial carcass, Gram-positive cocci belonging to the *Streptococcus*, *Rothia*, and *Kocuria* genera, frequently associated with animal mucosas, prevailed. In the contact surfaces of the dirty zone, such as the scalding tank and the de-hairing machine, we found a high abundance of *Anoxybacillus*, a thermophilic spore-forming bacterium. In the cutting plant and meat cuts at the beginning of their shelf life, we found dominance of Gram-negative proteolytic psychrotrophs, such as *Pseudomonas, Acinetobacter, Moraxella*, and *Psychrobacter,* while in meat cuts at the end of their shelf life, there was a high abundance of *Brochothrix*, a Gram-positive spoiler, and of various facultative anaerobic Gram-positive bacilli, including different members of *Leuconostoc*, *Latilactobacillus*, and *Carnobacterium.*

SourceTracker analyses suggest the notable influence of contact and non-contact surfaces on the microbiome of carcasses and meat cuts, which is particularly high in the carcasses at the end of the slaughter line and on the final products at the end of their shelf-life. However, it is important to note that the SourceTracker results assume source/sink relationships and therefore cannot account for directionality, meaning that the transfer of microorganisms could occur in either direction. Surfaces represented a possible source of *A. flavithermus, A. johnsonii, Psychrobacter* spp., *P. lundensis*, and *B. thermosphacta*, among other taxa. The prevalence of *A. flavithermus* on contact surfaces of the dirty zone, particularly in water samples from the scalding tank and the de-hairing machine, can be attributed to its thermotolerance [51, 52]. This enables this species to survive heat treatments during scalding and de-hairing, explaining its high relative abundance in the carcasses at the intermediate stage of the slaughter line (sampled following the singeing step). Other metataxonomic studies have also linked the presence of *A. flavithermus* in carcasses after singeing to its capacity to survive heat treatments [16].

The SourceTracker analysis also identified the cooling room and cutting plant surfaces as possible sources of *Psychrobacter* spp. in the meat cuts, which can be attributed to its capacity to grow at low temperatures and form biofilms on surfaces, thereby facilitating the colonisation of processing areas with varying nutrient levels [5, 49]. Conversely, the high abundance of *B. thermosphacta* and *P. lundensis* in the meat cuts may be attributed to the presence of these genera in the contact surfaces of

the cutting plant, such as knives and conveyors. Indeed, *B. thermosphacta* and *Pseudomonas* spp. have been previously observed to persist in meat processing environments [5, 50]. The packing conditions, including the use of protective atmospheres, may be significant factors driving microbial successions in the end products throughout their shelf-life. *B. thermosphacta* and lactic acid bacteria, such as *Leuconostoc* spp., are frequently dominant under conditions with less than 50% CO_2 and O_2, which were similar to the conditions used in the present study [51, 53].

The time of sampling was found to have an impact on the relative abundance of some genera, suggesting their capacity to grow, accumulate, and/or persist on facility surfaces. In the case of *Anoxybacillus*, the high abundance observed in the carcasses at the end of the morning working day (T2) can be attributed to its selection over the hours of scalding and de-hairing at high temperatures. Similarly, the large relative abundance of *Staphylococcus* and *Cutibacterium* in loins at the end of the morning workday (T2) could be attributed to the activity of operators, considering the typical occurrence of these taxa in the human skin microbiota [54, 55]. On the other hand, the high relative abundance at the end of the morning working day (T2) of *Moraxella* in sirloin at the beginning of its shelf-life and of *Brochothrix* and *Carnobacterium* at the end of loin's shelf-life could be related to their capacity to persist on the meat facility surfaces [5, 50]. Some biases due to sample storage must be considered carefully. For example, one of the main differences between carcass final and carcass cooling samples was the higher relative abundance of *Serratia*, a well-known psychrotolerant bacterium able to grow at low temperatures, in carcass cooling samples. On the other hand, the decrease of *Anoxybacillus* relative abundance during overnight cooling is in correspondence with its thermophilic nature. So, some changes due to the overnight conservation of swab samples from the first day of sampling must be expected.

Regarding MAGs analysis, as it was expected, some of the main species found at the read level were also found as the species with a greater number of MAGs obtained. The number of putative new taxa reflects the existence of still unknown microbial diversity in food production environments, as it was demonstrated recently in a global analysis of food metagenomes [56] and pig gut microbiomes [57]. In Holman et al. [57], the researchers found that 82% of the MAGs obtained were assigned to uncultured species, while in our study, we found that 51% of MAGs were uncultured or even unknown species. Due to technical issues with DNA extraction and the low microbial biomass in environmental samples, the majority of MAGs were obtained from carcass start samples, which were taken just after pig slaughtering and before the processing of the animal. The strain level analysis showed that some species, such as *A. flavithermus* and *A. johnsonii*, clearly spread in the meat industry, comprising several strains more or less adapted to different surfaces. On the other hand, species such as *M. maritypicum* and *L. reuteri* had several MAGs belonging to the same strain, with a clear adaptation to specific surfaces (on carcass intermediate for *M. maritypicum* and carcass start for *L. reuteri*). Nonetheless, this information must be interpreted with caution since the binning process may combine different strains into a single "consensus" strain. Additionally, further longitudinal studies with several sampling visits should be done to confirm the persistence of specific strains (even more than one strain from the same species), assessing their relatedness not only from a spatial point of view, as exposed in the current study, but also temporally. Finally, the main role of these strains in microbial successions must be addressed following the entire process of establishment and persistence on environmental surfaces.

Regarding the resistome, it was also found to be significantly influenced by the point of sampling and the processing room. Together with the lack of significant differences in the resistome profile among carcass and meat cut samples from different farms, this suggests that the facility surfaces may exert a greater impact on the resistome of the final products than the farm of origin. Further studies with a greater number of farms, selected considering their antimicrobial use, could provide greater insights into this matter. The resistome analysis demonstrated that the most prevalent ARGs in carcasses, meat cuts, and processing surfaces were associated with the same ARG families, linked to tetracycline, aminoglycoside, and lincosamide resistance. These findings support those from previous studies that had identified these ARG families as predominant in meat processing environments [6, 27, 28]. The high load of ARGs conferring resistance to tetracyclines may be attributed to the widespread use of antimicrobials from this class in pig farming. A metagenomic study conducted at Spanish pig farms identified ARGs from the tetracyclines and aminoglycosides families as the most prevalent in fecal, environmental, and slurry samples [58]. The occurrence of microorganisms resistant to these specific ARGs, in conjunction with the detection of ARGs of critical importance in carcasses and meat cuts, suggests that the meat production chain may act as a reservoir and transmission route to humans of a range of relevant antimicrobial resistance determinants.

The decrease in richness and diversity of ARGs found along the processing line is consistent with other metagenomic studies in slaughterhouses, where ARGs

levels in samples at the end of the processing line were relatively low [28, 59]. These findings indicate that processing procedures are effective in reducing ARGs loads. Nonetheless, it was noted that there was an increase in the ARGs load at two sampling points: the carcass after evisceration and the sirloin at the end of its shelf-life. The higher amount of ARGs observed in the carcass after evisceration (carcass final) in comparison to the carcass after peeling (carcass intermediate) is consistent with the higher load of ARGs observed in the surfaces of the clean zone as compared to those of the dirty zone. This may be attributed to the evisceration process, which may expose the carcasses and slaughterhouse surfaces to cross-contamination from the animal gut ecosystem. Furthermore, the increased load of ARGs observed in sirloin at the end of its shelf-life was linked to the increase of a single gene, *tet(L)*. While this gene was not associated with a specific genus, the likely reason for this increase is the growth and expansion of a specific microorganism carrying this gene during storage at refrigeration and modified atmosphere packaging. Similar findings in a metagenomic study were reported on chicken products, where the prevalence of specific ARGs varied depending on the packaging, reflecting the selection of different microorganisms throughout food processing and shelf-life [60].

The assembly-based analysis of the resistome linked the ARGs with specific genera, such as *Acinetobacter*, *Clostridium*, and *Streptococcus*, found in high abundance in the taxonomic analysis at the read level. The majority of ARGs identified on carcasses at the start of the slaughter line were associated with *Staphylococcus*. Some species within this genus are known to rapidly develop resistance to antibiotics, as evidenced by genomic studies of strains of *Staphylococcus* isolated from pigs reporting high resistance levels [61, 62]. Furthermore, ARGs linked to *Acinetobacter* were prevalent in carcasses, meat cuts, and facility surfaces, with a particularly high abundance on surfaces, which suggests the possibility that the facility surfaces may act as a source of *Acinetobacter* carrying ARGs contaminating meat and meat cuts. Meat processing surfaces could serve as reservoirs of *Acinetobacter* carrying ARGs [6], and we found that these ARGs were linked to resistance against aminoglycosides and beta-lactams on chromosomic contigs and tetracyclines on plasmidic contigs. Other reports have documented the presence in retail meat of multi-resistant *Acinetobacter* strains showing reduced susceptibility to tetracyclines, highlighting potential public health concerns [63, 64]. This underscores the possibility of the transmission of resistant *Acinetobacter* strains to the community via meat consumption and the transmission of ARGs to other strains or species.

Conclusions

To the best of our knowledge, the current study represents the first comprehensive metagenomic analysis of both the microbiome and resistome of two meat cuts throughout their processing line in a meat facility and during their shelf-life. The identification of significant variations in both the microbiome and resistome during processing highlights the effectiveness of metagenomic approaches in mapping microbial dynamics and successions in food industries and underscores the importance of understanding AMR sources and transmission routes in the food chain. In this regard, our findings indicated that the richness and diversity of bacteria and ARGs decreased as the products progressed through the production line. This suggests that the processing steps and the environments in which the industry operates exert a significant influence on the microbiome and resistome of carcasses and meat cuts. Furthermore, our findings indicated that meat can be a source of relevant ARGs, mainly related to the tetracycline family of antibiotics, followed by resistance to aminoglycosides and lincosamides, *Staphylococcus* and *Acinetobacter* being the main genera associated with the ARGs found.

Abbreviations

AMR	Antimicrobial resistance
ARGs	Antimicrobial resistance genes
MAGs	Metagenome-assembled genomes
WMS	Whole metagenome sequencing
PBS	Phosphate-buffered saline
NCBI	National Center of Biotechnology Information
CPM	Counts per million reads
LCA	Last common ancestor
ANI	Average Nucleotide Identity
MGEs	Mobile genetic elements
LSP	Lincosamides-Pleuromutilins-StreptograminA
MLS	Macrolides-Lincosamides-StreptograminB
OALSP	Oxazolidinones, Amphenicols, Lincosamides, StreptograminA and Pleuromutilins

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-025-02288-3.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

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Not applicable.

Authors' contributionS

JFC-D, HA, ML, MP, and AA-O designed the study. JFC-D, MO, AP, DB, HP, and HA performed the samplings. DNA extractions were performed by RC-G. Reads filtering was performed by JFC-D, and further computational and statistical analyses were performed by EF-T and JFC-D. EF-T, JFC-D, MO, and AA-O participated in the manuscript writing. All authors contributed to its revision and approved the final version.

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Data availability
Raw reads generated from the sequencing and analyzed during the current study are publicly available at the Sequence Read Archive of the National Center of Biotechnology Information (NCBI) under the BioProject number PRJNA1212716.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests

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