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Dynamics of microbiome and resistome in a poultry burger processing line

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A B S T R A C T

Traditionally, surveillance programs for food products and food processing environments have focused on targeted pathogens and resistance genes. Recent advances in high throughput sequencing allow for more comprehensive and untargeted monitoring. This study assessed the microbiome and resistome in a poultry burger processing line using culturing techniques and whole metagenomic sequencing (WMS). Samples included meat, burgers, and expired burgers, and different work surfaces.

Microbiome analysis revealed spoilage microorganisms as the main microbiota, with substantial shifts observed during the shelf-life period. Core microbiota of meat and burgers included *Pseudomonas* spp., *Psychrobacter* spp., *Shewanella* spp. and *Brochothrix* spp., while expired burgers were dominated by *Latilactobacillus* spp. and *Leuconostoc* spp. Cleaning and disinfection (C&D) procedures altered the microbial composition of work surfaces, which still harbored *Hafnia* spp. and *Acinetobacter* spp. after C&D. Resistome analysis showed a low overall abundance of resistance genes, suggesting that effective interventions during processing may mitigate their transmission. However, biocide resistance genes were frequently found, indicating potential biofilm formation or inefficient C&D protocols.

This study demonstrates the utility of combining culturing techniques and WMS for comprehensive of the microbiome and resistome characterization in food processing lines.

1. Introduction

Meat constitutes a vital component of the daily diet and its consumption has increased over the last 50 years. According to the Food and Agricultural Organization (FAO, 2023), 363.9 billion kg of meat were produced worldwide in 2023, with a per capita consumption of 45 kg. In particular, the poultry sector showed a production of 142.7 billion kg and a consumption of 14.9 kg/per capita (OECD, 2023), being the most produced and consumed meat worldwide. However, its high nutritional content, high water activity, and moderate pH make meat a highly perishable food commodity susceptible to the growth of both spoilage and pathogenic bacteria (Dave & Ghaly, 2011).

Spoilage microorganisms are responsible for altering organoleptic characteristics of food products (off-odors, off-flavors and slime formation) (Gram et al., 2002), shortening their shelf-life and causing food waste and economic losses (Ishangulyyev et al., 2019; Nychas et al., 2016). In addition, food producing animals are considered a reservoir of pathogenic microorganisms and they have been strongly associated with food-borne outbreaks. In 2021, 21.7 % of reported strong-evidence food-borne outbreaks in the European Union were associated with meat and meat products; 5.9 % were specifically associated with poultry meat (EFSA and ECDC, 2022). In addition, food processing environments (FPE) may also act as a source of pathogenic microorganisms which can cross-contaminate foods (Møretrø and Langsrud, 2017). Indeed, cross-contamination events have been frequently reported and associated with food-borne outbreaks (Thakali & MacRae, 2021).

Bacteria associated with poultry products and FPE may also carry antimicrobial resistance genes (ARGs), which could be transferred from one cell to another by horizontal transfer (Oniciuc et al., 2019), being particularly problematic if those ARGs were transferred to pathogenic bacteria. In fact, FPE are considered ideal environments for horizontal transfer events of ARGs (Bhatia et al., 2020; Kelly et al., 2009).

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Cleaning and disinfection (C&D) procedures are thought to prevent the presence of spoilage, pathogenic and persistant bacteria on FPE and, consequently, the cross-contamination from FPE to food products (Agüeria et al., 2021). However, it has been demonstrated that long-term exposure to subinhibitory concentrations of biocides can select for bacteria with enhanced resistance to biocides and even to antibiotics (Buffet-Bataillon et al., 2016; Webber et al., 2015).

Due to these factors, surveillance programs should monitor both food products and FPE.

Traditionally, culturing techniques along with molecular techniques have been used to monitor a small, targeted number of pathogenic microorganisms or ARGs based on their threat level (Allard et al., 2018). However, incorporating untargeted approaches, such as whole metagenome sequencing (WMS), could be advantageous, as they provide a more complete profile of antimicrobial resistant bacteria (both spoilage and pathogenic) and the ARGs present in the sample (Doyle et al., 2017).

Most WMS studies have focused on animals or farm environments, but only a limited number of WMS studies have focused on food products and FPE (Alvarez-Molina et al., 2023; Cobo-Díaz et al., 2021; Li et al., 2020; Oniciuc et al., 2024; Valentino et al., 2022). Notably, no previous study has examined poultry FPE at a secondary production setting (i.e. industry level). In this sense, this study contributes to this expanding field by a) assessing the microbial dynamics throughout the burger processing line by combining culturing techniques and WMS; b) determining the potential role of the burger processing line in the selection and dissemination of antimicrobial resistance, and c) assessing the influence of the microbiome and resistome of the FPE on the microbiome and resistome of the final product.

2. Material and Methods

2.1. Sampling strategy

This study was conducted in a burger processing line of a poultry processing plant. Different poultry products and food-contact surfaces were sampled during different production cycles on two different production days. The poultry products analyzed comprised chicken thighs, packaged burgers, and expired packaged burgers. The expired burgers were kept at 0–4 °C in a modified atmosphere (70 % N_2 and 30 % CO_2) until five days past the sell-by date, for a total storage time of 27 days. Food-contact surfaces included different equipment: formula table, grinder, kneader, forming hopper and conveyor belt. These food-contact surfaces were sampled before the start of the production, when they were still clean following the C&D procedure (referred to as clean surfaces), and during processing (referred to as utilized surfaces).

2.2. Sample collection

Five chicken thighs and four burger packages were collected per production cycle and day using sterile plastic bags. Two of the four burger packages were maintained under cooling conditions until five days after the sell-by date in order to obtain the expired burger samples. Clean and utilized surfaces were sampled using sterile sponge swabs (Nasco Whirl-Pak Speci-Sponge, Wisconsin, USA) pre-moistened with 20 mL of sterile buffered peptone water (BPW) (Oxoid, Basingstoke, United Kingdom). Around 1 m^2 was sampled from each clean surface and 300 cm^2 (0.03 m^2) from each utilized surface to obtain sufficient DNA. Swabs from all clean surface samples were placed in the same bag, obtaining a pool of clean surfaces per production day (a total of two pools). Likewise, swabs from all utilized surface samples were placed in the same bag, obtaining a pool of utilized surfaces per production cycle and day (a total of seven pools). Once collected, all sample bags were transported to the laboratory in a cooling box containing ice packs.

2.3. Sample processing

Once in the laboratory, samples were processed to carry out the following analysis (culturing and WMS). For poultry products, 25 g of each sample were mixed with 225 mL of BPW in blender bags (VWR, Pennsylvania, United States) and then homogenized at 230 revolutions per minute (rpm) during 1 min in a peristaltic homogenizer (Stomacher 400 Circulator, Seward, New York, United States). For utilized surfaces, all the sponges from the same pool were mixed with 100 mL of BPW in filter bags and then homogenized at 230 rpm during 30 s. However, no BPW was added to clean surface sponges to avoid DNA dilution during the homogenization step. After the homogenization step, a stock dilution of each sample was obtained.

2.4. Culturing techniques

In order to evaluate the microbiota throughout the burger processing line, mesophiles, psychrotrophes, *Pseudomonadaceae*, *Enterobacterales* and lactic acid bacteria (LAB) were cultured according to UNE-EN ISO standards. Briefly, Plate Count Agar (tryptone 5.0 g/L, yeast extract 2.5 g/L, glucose 1.0 g/L, agar 9.0 g/L) was used for mesophiles and psychrotrophes and plates were incubated at 30 °C for 72 h and at 7 °C for 10 days, respectively; *Pseudomonas* CFC/CN agar enriched with Cephalothin, Fucidin, Cetrimide (CFC) selective supplement was used for *Pseudomonadaceae* and plates were incubated at 25 °C for 48 h; Violet Red Bile Glucose Agar (VRBG) was used for *Enterobacterales* and plates were incubated at 37 °C for 24 h; De Man Rogosa Sharpe Agar (MRS) and M−17 agar were used for LAB and plates were incubated for 30 °C/3–5 days under anaerobic conditions. A more detailed description of the culturing method of these microbial groups and the UNE-EN ISO standards can be found in a published book chapter (Merino et al., 2021).

2.5. Whole metagenomic sequencing (WMS)

2.5.1. DNA extraction and sequencing

DNA extraction was carried out from stock dilutions of the different poultry products and surfaces (section 2.3.), but also from *Enterobacterales* plates scrapes (section 2.4.) to specifically characterize the enterobacteria resistome. Both stock dilutions and plates scrapes were collected and centrifuged at 3345 × g for 10 min at 4°C. The supernatants were removed and the pellets were recovered and stored at − 20 °C. DNA extraction was performed following the manufacturer's instructions of the PowerSoil Pro Kit (Qiagen, Hilden, Germany), but performing a double elution step with 25 μL to improve the DNA yield. Then, DNA concentrations were measured using a Qubit fluorometer and following the Qubit 1x dsDNA HS assay kit protocol (Thermo Fisher Scientific, Massachusetts, United States).

Metagenomic libraries were prepared using the Nextera XT library construction (San Diego, CA, USA) and WMS was performed on the Illumina NovaSeq 6000 platform, generating 2 × 151 bp reads at a mean sequencing depth of 3 Gb.

2.5.2. Reads pre-processing

Reads were processed with KneadData v.0.7.4 (https://huttenhower.sph.harvard.edu/kneaddata/) to remove adaptor sequences and low quality bases with Trimmomatic v.0.33 (Bolger et al., 2014), as well as to remove poultry-derived sequences by alignment to the poultry genome (GenBank assembly accession: GCA_000002315.5) with bowtie2 v.2.4.5 (Langmead & Salzberg, 2012). Finally, FastQC v.0.11.9 was employed to obtain a quality control report. After quality trimming and hosts sequences removal, the mean sequencing depth was ~ 5Mreads. Due to the low bacterial load and/or the high host DNA content, there were two samples with fewer than 200,000 reads (samples CS_2 and B_2.3). All samples, including those with fewer than 200,000 reads, were used for the analysis.

2.5.3. Taxonomic analysis

Taxonomical assignment of reads was done with Kraken2 v.2.1.3 (Wood et al., 2019) using the kraken2-microbial standard database. For the scope of this article, only bacterial reads were analyzed and used to calculate ecological metrics. Then, Bracken v.2.9 (Lu et al., 2017) was employed to estimate relative abundances using Kraken2 classification results.

The presence of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) was also evaluated by Kraken2. To determine the presence or absence of ESKAPE pathogens, a threshold of 1,000 reads was set, helping to prevent false positives in samples with low sequencing depth. Consequently, samples were considered "positive" when the number of reads assigned to ESKAPE pathogens was $\geq$ 1,000, "present at trace levels" when the number of reads was between 100 and 1,000, and "negative" when the number of reads was $<$ 100.,

2.5.4. Resistome analysis

ShortBRED v.0.9.4 (Kaminski et al., 2015) was employed to identify resistance genes from the trimmed and filtered metagenomic reads using a marker database generated from the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2023). CARD includes disinfectants and antiseptics resistance genes (Alcock et al., 2023), which is worth noting as these antimicrobials are commonly employed in food industries. Genes identified in ShortBRED are quantified in Reads Per Kilobase per Million mapped reads (RPKM).

2.5.5. Plot generation and statistical analysis

GraphPrism was used to generate microbial counts plots as well as to compare sample types via analysis of variance (ANOVA) followed by *post-hoc* Tukey test; differences were considered statistically significant if p value $\leq$ 0.05.

Statistical differences in taxonomic relative abundances of the most abundant genera (those representing $>$ 80 % of the total bacterial relative abundance) across different sample type groups were assessed through the Wilcoxon test using the function "wilcox.test" in R v3.6.1. Additionally, α-diversity and β-diversity analysis of both microbiome and resistome were carried out at species level using the *vegan* package v2.5.7 in R (Oksanen et al., 2022); Shannon diversity was calculated using the function "diversity", while the Bray-Curtis dissimilarity matrix was generated using the function "vegdist". This was then used as input for Principal Coordinates Analysis (PCoA). Statistical differences in α-diversity and β-diversity among the different sample type groups were calculated through the permutational multivariate analysis of variance (PERMANOVA) using the "adonis" function. Finally, a correlation matrix among bacterial genera and ARGs was constructed using Halla v.0.8.20 (Ghazi et al., 2022) by calculating all possible pairwise Spearman's correlations. Only correlations with the p value $<$ 0.05 were considered statistically robust. Prior to running Halla, the least abundant genera, representing 1 % of the total bacterial relative abundance were discarded. To achieve this, genera were sorted based on their relative abundance, cumulative sums were calculated, and genera whose cumulative sum was less than 1 % were removed.

2.5.6. Data availability

Filtered reads are available on the Sequence Read Archive of the National Center of Biotechnology Information (NCBI) under the accession number PRJNA1095416.

3. Results

3.1. Culture-based analysis of samples from the poultry burger processing line showed differences in microbial abundance and predominance

Expired burger samples showed the highest mesophiles and psychrotrophes counts (mean value of 8.74 and 8.75 Log_{10} CFU/g, respectively), which were significantly higher (ANOVA, $p < 0.01$) than those of meat (mean value of 6.72 and 7.17 Log_{10} CFU/g, respectively) and burger samples (mean value of 6.02 and 5.50 Log_{10} CFU/g, respectively) (Fig. 1A). In contrast, clean surface samples showed the lowest microbial counts. Indeed, C&D procedures achieved a 4-Log_{10} reduction in mesophiles and psychrotrophes counts and a reduction of *Pseudomonadaceae*, *Enterobacterales* and LAB counts until below the detection limit (Fig. 1B).

Pseudomonadaceae had the highest microbial counts in meat and burger samples (mean value of 6.52 and 5.58 Log_{10} CFU/g, respectively), followed by LAB, and then *Enterobacterales*. Although the counts of these microbial groups were slightly higher in meat than in burger samples, the most significant changes were observed after the shelf-life period. Both spherical-shaped and rod-shaped LAB counts were significantly higher (ANOVA, $p < 0.01$) in expired burgers, reaching microbial that exceed the critical spoilage threshold (Jones, 2004) (8.76 Log_{10} CFU/g and 8.52 Log_{10} CFU/g, respectively). In contrast, *Pseudomonadaceae* counts on expired burgers were significantly lower (ANOVA, $p <$ 0.01) than those on meat and burger samples (Fig. 1A).

3.1.1. A drastic shift in the diversity and composition of burger samples microbiomes was observed after the shelf-life period

In agreement with our culture-based results, PCoA of Bray-Curtis dissimilarities showed significant discrimination (adonis test, $p <$ 0.001) between expired burger samples and meat and burger samples, with expired burger samples belonging to a completely different cluster from meat and burger samples (Bray-Curtis distance between burgers and expired burgers of 0.996) (Fig. 2). Shannon index values of meat and burger samples were significantly higher than those of expired burger samples, indicating higher microbial diversity in meat and burger

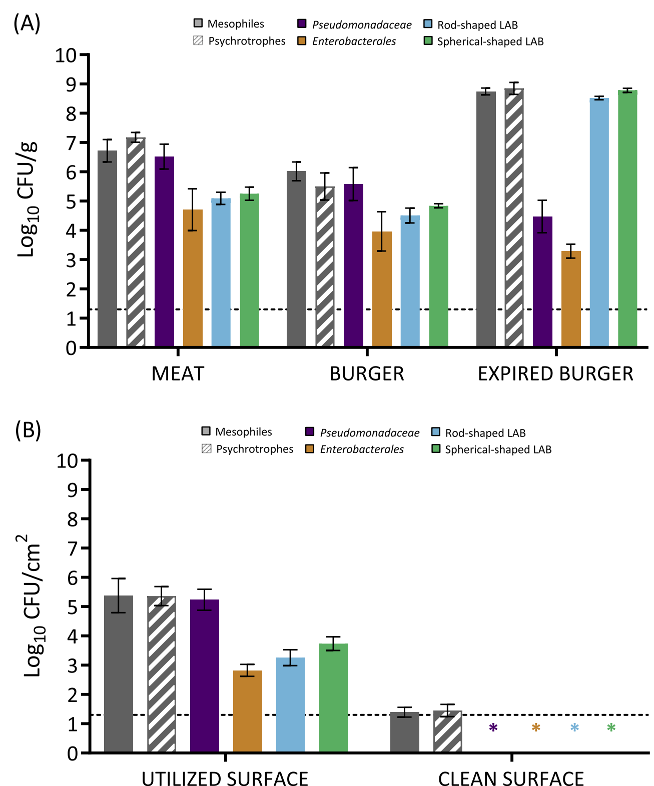

Fig. 1. Microbial counts throughout the burger processing line. Mesophiles (■), psychrotrophes (▨), *Pseudomonadaceae* (■), *Enterobacterales* (■), rod-shaped lactic acid bacteria (■), and spherical-shaped lactic acid bacteria (■) counts in different poultry products (A) and surfaces (B) throughout the processing line. Horizontal dotted line represents the limit of detection. * microbial counts are below the detection limit.

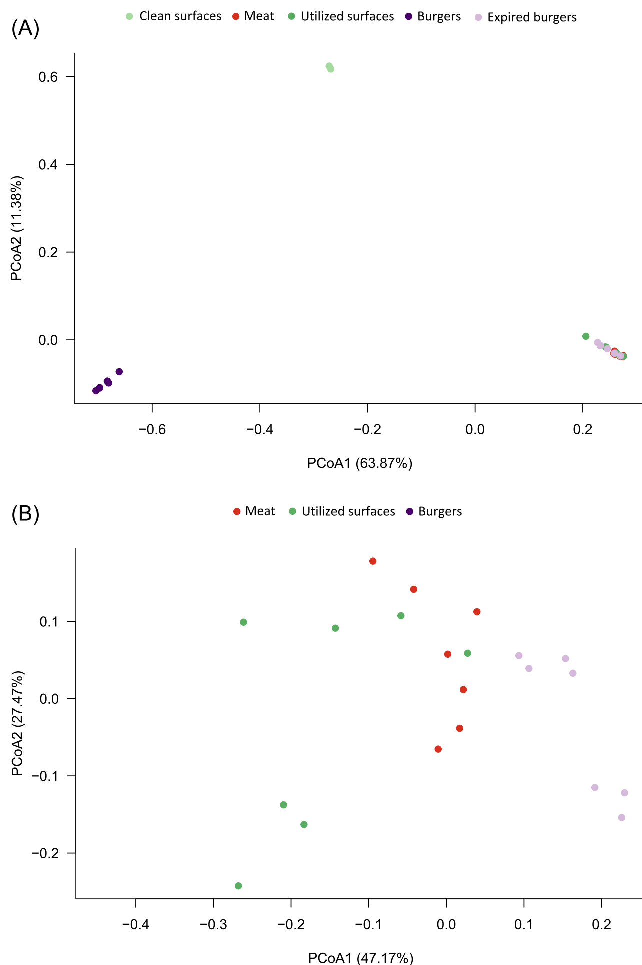

Fig. 2. Beta diversity analysis of the microbiomes of the different sample type groups throughout the burger processing line. PCoA based on the Bray-Curtis dissimilarities performed on the species-level bacterial profiles obtained with Kraken2. Fig. 2A includes all sample type groups, while Fig. 2B excludes clean surface samples and expired burger meat samples to better appreciate the differences among the rest of sample type groups. Points are color-coded according to the sample type (CS: Clean surface samples (●); M: Meat samples (●); S: Utilized surface samples (●); B: Burger samples (●); EB: Expired burger samples (●)).

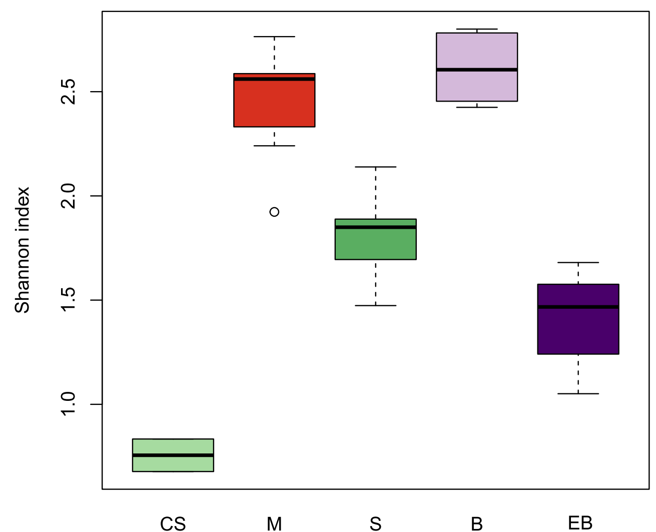

Fig. 3. Alpha diversity analysis of the microbiomes of the different sample type groups throughout the burger processing line. Shannon index boxplot of the different sample type groups performed on the species-level bacterial profiles obtained with Kraken2. Boxplots are color-coded according to the sample type (CS: Clean surface samples (■); M: Meat samples (■); S: Utilized surface samples (■); B: Burger samples (■); EB: Expired burger samples (■)).

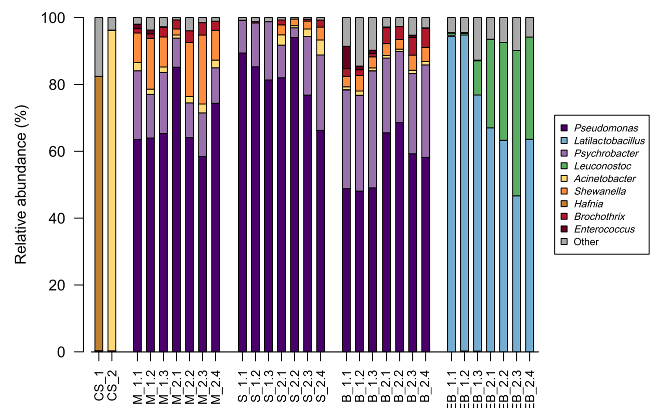

Fig. 4. Microbial composition throughout the burger processing line. Relative abundances of the main microbial genera in different samples throughout the burger processing line. CS: Clean surface samples; M: Meat samples; S: Utilized surface samples; B: Burger samples; EB: Expired burger samples. The first number of the sample code corresponds to the production day and the second number corresponds to the production cycle.

samples than in expired burger samples. The Simpson index values (Figure S2) and the number of observed species (Figure S3) support similar conclusions, thereby reinforcing the observations derived from the Shannon index analysis. In contrast, there was no significant difference in alpha diversity comparing meat and burger samples (Fig. 3).

As observed from our culture-based approach, meat and burger samples were dominated by *Pseudomonas* spp., *Psychrobacter* spp., *Shewanella* spp. and *Brochothrix* spp., while expired burger samples were dominated by LABs *Latilactobacillus* spp. and *Leuconostoc* spp. (Fig. 4). It should be noted that the relative abundance of *Leuconostoc* spp. was higher in expired burgers on the second sampling day compared to the first sampling day. Though meat and burger samples harbored a similar range of bacterial genera (Fig. 4), they were compositionally distinct (Fig. 2B; adonis test, $p < 0.01$). The relative abundance of *Psychrobacter* spp. was significantly higher in burger samples (Wilcoxon test, $p < 0.05$), while the relative abundance of *Shewanella* spp. was significantly higher in meat samples (Wilcoxon test, $p < 0.05$). Additionally, the relative abundance of *Pseudomonas* spp. increased in burgers sampled on the second day compared to those from the first day.

3.1.2. Cleaning and disinfection procedures altered the diversity and the composition of surface samples microbiomes

PCoA of Bray-Curtis dissimilarities revealed that clean surface samples formed a distinct cluster, completely separate from utilized surface samples. The utilized surface samples were closer in proximity to the clusters of meat and burger samples (Fig. 2A).. In addition, the low Shannon index values of clean surface samples (<0.5) (Fig. 3) revealed that C&D procedures caused a significant decrease in microbial diversity. The Simpson index values (Figure S2) and the number of observed species (Figure S3) support similar conclusions, thereby reinforcing the observations derived from the Shannon index analysis.

Utilized surfaces were dominated by taxa present in meat and burger samples (Fig. 4), which was expected as they were sampled during processing. This aligned with culturing techniques results, as utilized surface samples showed similar microbial counts as observed for meat and burger samples. In contrast, clean surfaces harbored *Acinetobacter* spp. and *Hafnia* spp., which were not detected in any of the other sample

types.

3.2. ESKAPE pathogens were detected in different samples throughout the burger processing line

E. faecium (Fig. 5A) and *K. pneumoniae* (Fig. 5B) were present at trace levels in samples throughout the processing line (100–1,000 assigned reads), while *S. aureus* and *Enterobacter* spp. were not detected in any sample (<100 assigned reads) (data not included in Fig. 5). *A. baumannii* and *P. aeruginosa* were the only ESKAPE pathogens detected in the burger processing line (>1,000 assigned reads). *A. baumannii* was detected in two utilized surfaces at a relative abundance of 0.04 % and 0.05 % (Fig. 5C); and *P. aeruginosa* was detected in all meat samples from the second sampling day, and in all utilized surface samples from both sampling days, ranging from 0.07 % to 0.12 % (Fig. 5D).

Although the presence of the ESKAPE pathogen *E. faecium* could not be confirmed (<1,000 assigned reads in all samples), *Enterococcus faecalis* was detected in three samples throughout the processing line (two surface samples and one burger sample), ranging from 0.02 % to 3.07 % (data not shown).

3.3. Resistome profile throughout the burger processing line

To assess the potential role of the poultry burger processing line in the selection and dissemination of antimicrobial resistance, the resistome profile of the burger processing line was determined by WMS. Significant differences in ARGs composition and abundance were observed among the different sample type groups (Fig. 6A and 6B). ARGs conferring resistance to different drug classes were detected in meat and utilized surface samples, with ARGs conferring resistance to biocides (*qacE* and *qacL*) present at the highest abundance, followed by ARGs conferring resistance to tetracyclines and aminoglycosides (Fig. 6A and 6B). A completely different ARG composition was observed in expired burger samples, with ARGs conferring resistance to lincosamides (*lnuA* and *lnuE*) in highest abundance, followed by ARGs conferring resistance to tetracyclines, which were only detected in expired burgers from the second sampling day. Expired burgers exhibited significantly less ARG diversity than meat and utilized surfaces. This result revealed that the shelf-life period not only altered the composition and diversity of sample microbiomes, but also of sample resistomes.

A total of 56 ARGs were detected throughout the burger processing line (Fig. 6B). Of these, 6 ARGs (*tetM*, *lnuA*, *aph(6)-Id*, *aadA2*, *aadA5* and *aadA27*) belonged to Rank I or Rank II ARG families (Zhang et al., 2021), so they are considered high-risk for being human-associated and mobile. *lnuA* was detected in high abundance in all expired burger samples, while lincosamide ARGs, including *aph(6)-Id* and *aadA27*, were detected in different meat and surface samples. In addition, *tetM* and *sul1*, both detected in different meat and surface samples, were also considered ARGs of high clinical concern by the World Health Organization (WHO, 2019). Besides these high-risk ARGs, it is also worth noting that the colistin resistance gene *mcr*-4.2 was detected in different meat and utilized surface samples.

3.3.1. Co-occurrence of Pseudomonas spp. And biocide resistance genes as well as LAB and lincosamides resistance genes

Though in the present study it was not possible to assign ARGs to taxa

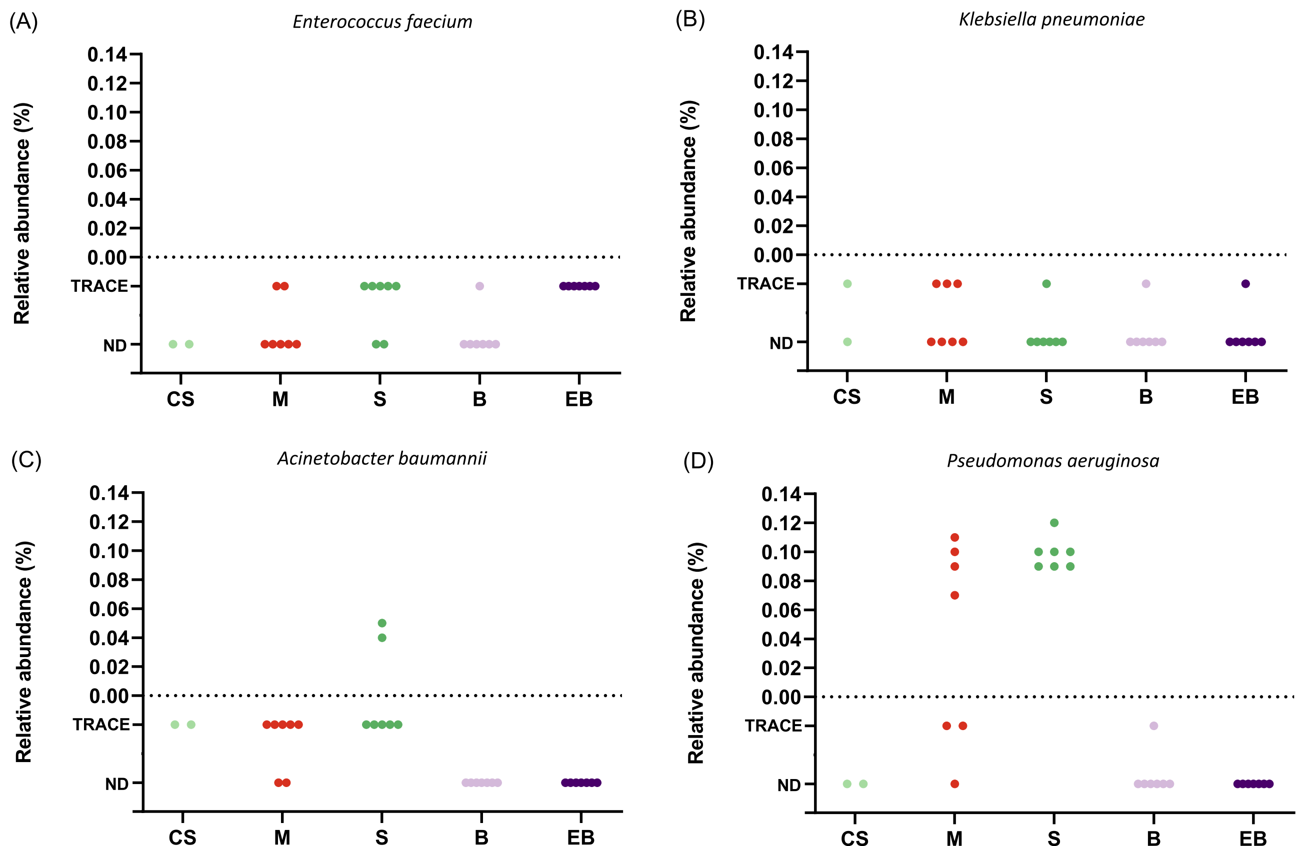

Fig. 5. Detection of ESKAPE pathogens throughout the burger processing line. Relative abundance of the ESKAPE pathogens detected or probably detected by Kraken2 (*Enterococcus faecium* (A), *Klebsiella pneumoniae* (B), *Acinetobacter baumannii* (C) and *Pseudomonas aeruginosa* (D)) in different samples throughout the burger processing line (CS: Clean surface samples (●); M: Meat samples (●); S: Utilized surface samples (●); B: Burger samples (●); EB: Expired burger samples (●)).Those ESKAPE pathogens that were not detected (*Staphylococcus aureus* and *Enterobacter* spp.) are not included in this figure. ESKAPE pathogens were not detected (ND) when < 100 reads were assigned to them, detected at trace levels when 100–1,000 reads were assigned to them and detected when > 1,000 reads were assigned to them.

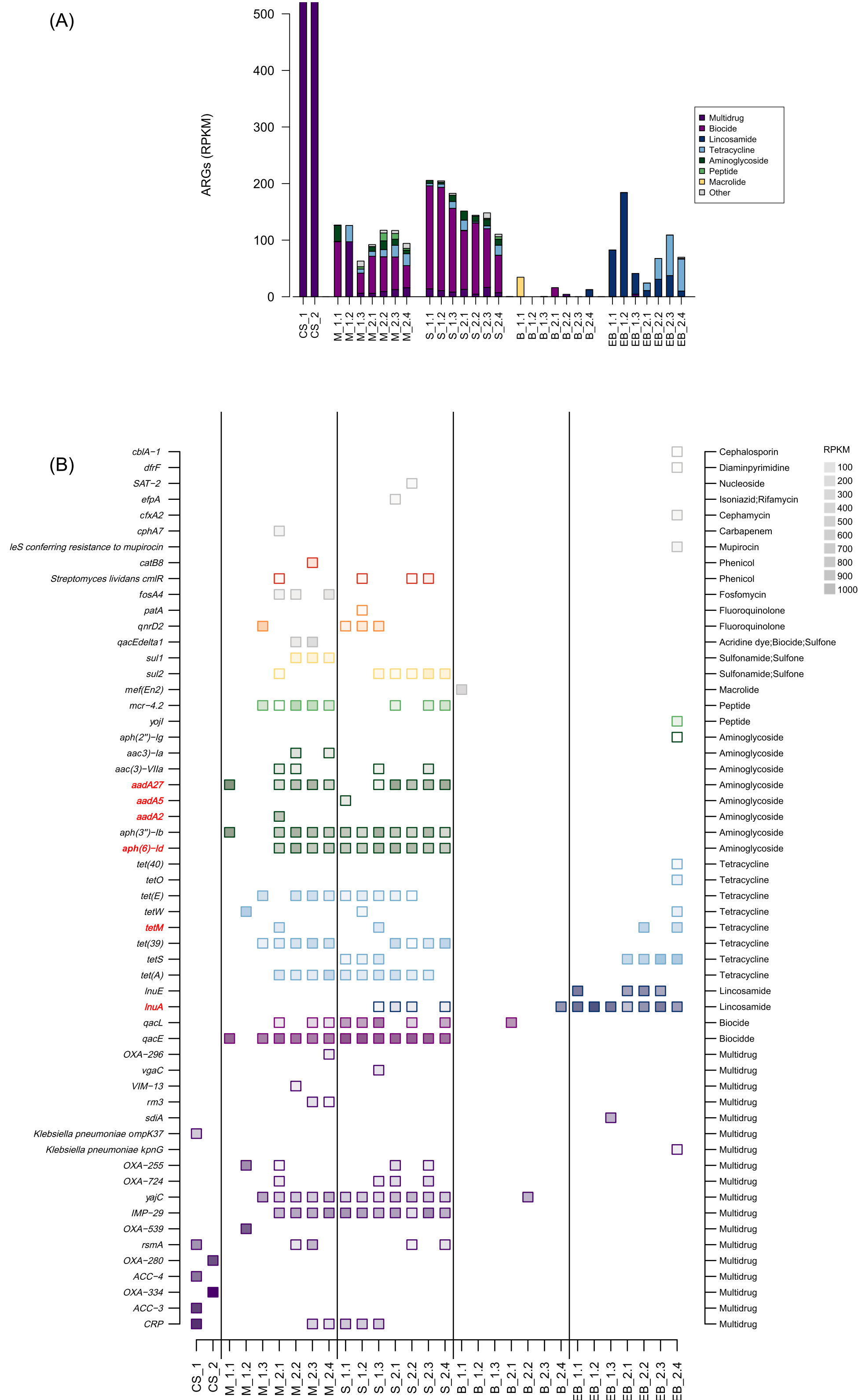

Fig. 6. Resistome profile throughout the burger processing line. Normalized abundance of drug classes (A) and ARGs (B) in the different samples throughout the burger processing line. CS: Clean surface samples; M: Meat samples; S: Utilized surface samples; B: Burger samples; EB: Expired burger samples. The first number of the sample code corresponds to production day and the second number corresponds to the production cycle. Red color indicates high-risk ARGs. Different colors represent different drug classes. Color gradient indicates ARG abundance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

given the difficulty in assembling short read data, we sought to correlate ARG and taxa abundances as an approximation. Comparison between microbial relative abundance and ARGs normalized abundance throughout the different samples of the burger processing line allowed us to determine significant associations (Fig. 7) between the main genera and ARGs and, consequently, to confirm the fact that resistomes were strongly determined by microbiomes. *Pseudomonas* spp., the predominant genus in meat, utilized surface and burger samples, was the taxonomic group associated with the highest number of ARGs (9 ARGs), including biocide ARGs (*qacE* and *qacL*) and aminoglycoside ARGs (*aph(6)-Id* and *aph(3)-Ib*), among others. Similarly, a strong correlation ($p < 0.01$) was also observed between LAB (*Latilactobacillus* spp., *Lactiplantibacillus* spp., *Paucilactobacillus* spp., *Leuconostoc* spp. and *Weisella* spp.) and lincosamide ARGs (*lnuA* and *lnuE*), being only detected in expired burgers.

3.3.2. Clean surfaces could be a reservoir of ARGs and contribute to AMR dissemination

Different beta-lactams ARGs (*ACC-3*, *ACC-4*, *CRP*, *OXA-280* and *OXA-334*) were detected in clean surface samples (Fig. 6B), revealing that C&D procedures failed to remove all antimicrobial resistant bacteria.

In addition, the association analysis between taxa and ARGs (Fig. 7) revealed that *Hafnia* spp. and *Yersinia* spp., the main genera in clean surface samples from the first sampling day, were strongly correlated ($p < 0.01$) with *ACC-3* and *ACC-4* ARGs. In order to investigate the remaining bacterial community on surfaces after C&D procedures, culturing enrichment in an *Enterobacterales*-specific medium was carried out prior to DNA extraction and sequencing to determine whether the ARGs detected in clean surface samples were also present in other samples (Fig. 8). Several ARGs were observed in all samples for which we detected one or more *Escherichia coli* strains with StrainGE (data not shown), suggesting ubiquity within this species. Since any associations found with these ARGs would likely be driven by *E. coli* presence alone, we excluded them from this analysis. For *Enterobacterales* ARGs detected in clean surface samples, *ACC-3* and *ACC-4* were also detected in different meat, utilized surface, burger and expired burger samples from different batches and sampling days, suggesting that clean surfaces might serve as a potential source of beta-lactams ARGs, which could be transferred to food products, potentially contributing to the spread of antibiotic resistance through the food supply chain.

It is also worth noting that *Acinetobacter* spp., the main genus in clean surface samples from the second sampling day, was strongly correlated ($p < 0.01$) with *mcr*-4.2, *tet(39)* and *aadA27*.

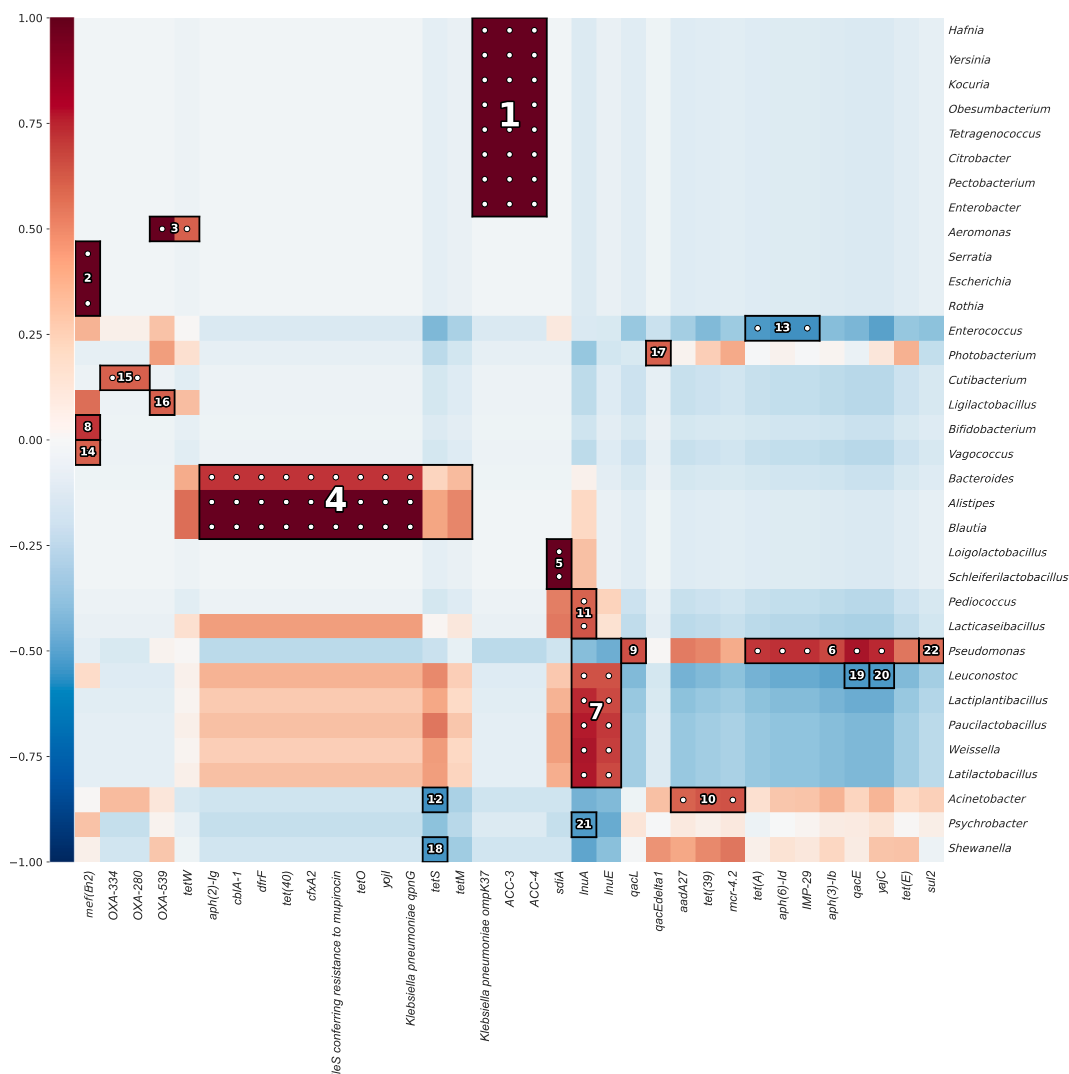

Fig. 7. Co-occurrence of taxa and ARGs. Associations between taxa and ARGs based on Spearman correlation. The least abundant genera, representing 1% of the total bacterial relative abundance, were not included (see Material and Methods). The color gradient indicates whether the Spearman correlation is positive (values above 0) or negative (values below 0). Statistically significant associations are depicted blocks, with numbers within the blocks representing the order of significance (where 1 is the most statistically significant).

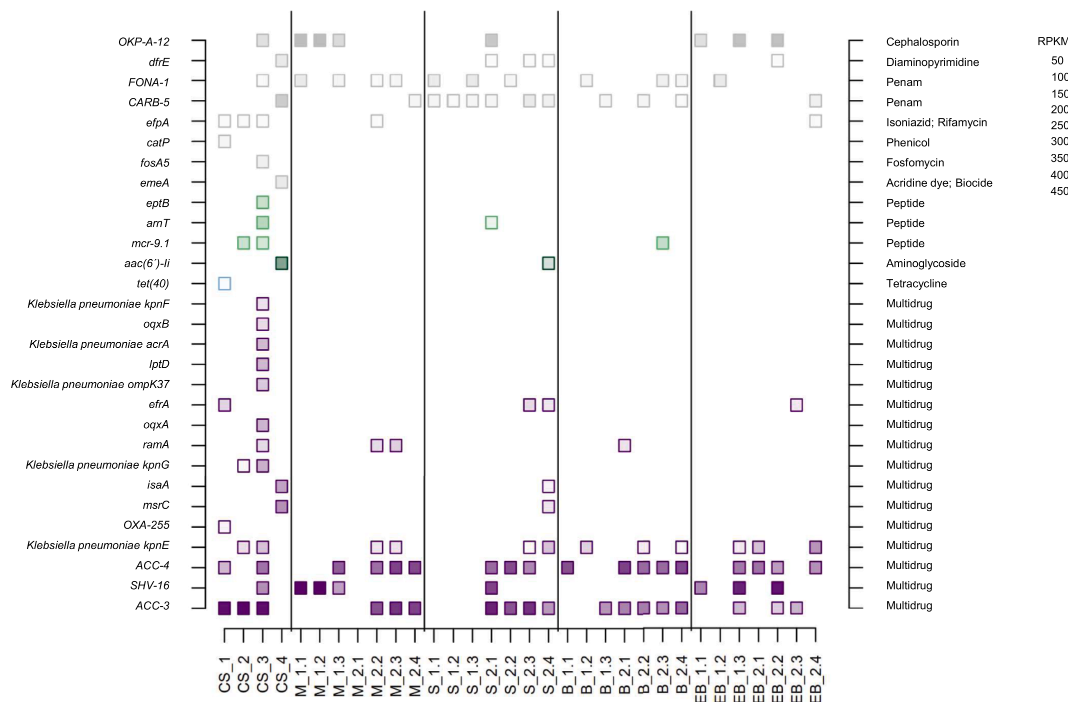

Fig. 8. Detection of *Enterobacterales* ARGs found on clean surfaces in different sample type throughout the burger production line. Normalized abundance of clean surface ARGs in the different samples throughout the burger processing line. ARGs determination was carried out with those samples in which culturing enrichment in an *Enterobacterales*-specific medium was carried out before DNA extraction. Ubiquitous ARGs of *Escherichia coli* were not included (see Results). CS: Clean surface samples; M: Meat samples; S: Utilized surface samples; B: Burger samples; EB: Expired burger samples. The first number of the name code corresponds to the production day and the second number corresponds to the production cycle. Different colors represent different drug classes. Color gradient indicates ARG abundance.

4. Discussion

In the current study, a combination of culturing techniques and WMS was employed to assess the microbiome of a burger processing line. While culturing techniques provide valuable information regarding the absolute abundance of different bacterial taxa, they are typically laborious, expensive, and time-consuming. Furthermore, they underestimate microbial diversity, since they are unable to detect fastidious and unculturable microorganisms (Forbes et al., 2017; Mayo et al., 2014). In contrast, WMS provides a comprehensive view of microbial composition and diversity. However, some limitations of this technique include a) the potential sequencing of DNA from dead cells, which leads to the possibility of identifying taxa that are not actively viable (Pennone et al., 2022); and b) the high abundance of host DNA (in this case, poultry DNA), which dilutes microbial DNA, and consequently, reduces the sensitivity of detecting less abundant microorganisms. Hence, the combination of both techniques was used to obtain complementary information and to overcome and compare the limitations inherent in each individual technique.

Results of culturing techniques revealed slight differences in terms of bacterial abundance between meat and burger samples and great differences in terms of both bacterial abundance and predominance between burger and expired burger samples, demonstrating that factors such as meat transformation and shelf-life period may shape poultry products microbiomes, as previously reported (Dourou et al., 2021; Li et al., 2020; Wang et al., 2020). In addition, great differences between clean and utilized surfaces were also evident, highlighting the role of C&D procedures in reducing the microbial load of food-contact surfaces (Møretrø and Langsrud, 2017), but failing to remove the entire microbial community.

Based on WMS analysis, the microbial composition of meat samples was consistent with previously published articles (Dourou et al., 2021; Li et al., 2020; Rouger et al., 2018), with *Pseudomonas* spp., *Psychrobacter* spp., *Shewanella* spp. and *Brochothrix* spp. representing the core microbiota. Li et al., 2020 also reported *Pseudomonas* spp. and *Shewanella* spp. as the predominant genera in chicken breasts samples. This was not surprising as *Pseudomonas* spp. is considered the main spoilage genus of poultry meat stored under chilling and aerobic conditions (Dourou et al., 2021). More specifically, *Pseudomonas fragi* is considered one of the most prevalent species in poultry meat samples (Marmion et al., 2021), which aligns with the results obtained in this study (Figure S1). Similarly, *Shewanella* spp. is a closely related genus to *Pseudomonas* spp. which also contributes to meat spoilage (Doulgeraki et al., 2012). In fact, *Shewanella putrefaciens*, also present in samples of this study, is responsible for off-odours and meat degradation (Nychas et al., 2008). Another abundant microorganism was *Brochothrix thermosphacta* (Figure S1), commonly associated with the spoilage of fresh meats (Ercolini et al., 2011; Pennacchia et al., 2011) and reported at higher rates in chicken legs stored under different atmospheric conditions (Rouger et al., 2018). Finally, the presence of *Psychrobacter* spp. was also observed in poultry meat stored under aerobic conditions, although it is considered a mild-spoilage microorganism (Dourou et al., 2021).

The microbial composition and diversity of burger samples reflected that of meat samples, which was not surprising since none of the meat transformation steps was intended to alter the poultry meat microbiota. However, beta-diversity analysis revealed statistical differences between these two sample types, driven by statistical differences in *Psychrobacter* spp. and *Shewanella* spp. relative abundances. These differences could be attributed to different factors, such as the contact of meat with surfaces or the incorporation of other ingredients.

The greatest significant shift was observed after the shelf-life period, demonstrating the significant influence of storage conditions on the composition and diversity of burger microbiomes. Although the predominant genus in burgers was *Pseudomonas* spp, their presence was not detected in expired burgers, probably due to the low oxygen packaging conditions which could impair their growth. Expired burgers were

dominated by *Latilactobacillus* spp. and *Leuconostoc* spp, and specifically by *Latilactobacillus sakei* and *Leuconostoc gelidum* (Figure S1), which are considered important competitors in meat under chilling and vacuum or modified atmosphere packaging (Doulgeraki et al., 2012). In fact, they have been detected in chilled marinated broiler strips packaged under modified atmosphere (Nieminen et al., 2012). Alpha diversity analysis demonstrated expired burger samples were significantly less diverse than freshly produced burger samples. In the study carried out by Dourou et al. (2021) a significant decrease in the bacterial diversity after the storage time was also observed, suggesting that a fraction of the total bacteria population dominated and was responsible for food spoilage (Nychas & Skandamis, 2005). In addition to examining the microbiome and resistome for food safety, it is crucial to consider the potential impact of microbiome composition on sensory attributes such as taste, appearance, and texture. Spoilage microorganisms can significantly affect these qualities, leading to off-odors, off-flavors, and undesirable textures (Nychas et al., 2008; Zhu *et al.*, 2022). For instance, the dominance of *Latilactobacillus* spp. and *Leuconostoc* spp. in expired burgers can result in sour flavors and changes in texture, while the presence of *Pseudomonas* spp. in fresh meat is associated with the development of off-odors (Casaburi et al., 2015). Therefore, understanding the microbiome dynamics is essential not only for ensuring food safety but also for maintaining food quality during storage.

Clean surface samples showed a completely different microbiome than utilized surface samples, being dominated by *Acinetobacter* spp. and *Hafnia* spp. It has been reported that *Acinetobacter* spp. and different genera belonging to the *Enterobacterales* order, such as *Hafnia* spp., showed a great tolerance to low temperatures, low nutrients availability, and cleaners and disinfectants (Møretrø and Langsrud, 2017), so it was expected to find these genera on surfaces just after C&D procedures. In addition, this discrimination between clean and utilized surfaces was also observed in alpha diversity analysis, showing utilized surfaces a significantly higher diverse microbiome than clean surfaces. Hence, stressful environmental conditions caused by C&D procedures altered the diversity of surface sample microbiomes, differing from the studies carried out by Valentino et al., 2022 and Møretrø and Langsrud, 2017, where C&D procedures did not alter the presence of a high diverse microbiome in clean surfaces.

Although WMS has not been widely used for risk assessment, the detection and characterization of pathogens by this technique has been previously carried out in different meat products and meat processing environments (Li et al., 2020; Yang et al., 2016). In the present study, different pathogens (*A. baumannii*, *P. aeruginosa* and *E. faecalis*) were detected in different meat and surface samples throughout the burger processing line. Although their detection did not definitively confirm the presence of live and infectious pathogenic cells, it indicated the occurrence of pathogens in the burger processing line. In addition, ESKAPE pathogens are of particular concern in healthcare settings due to their resistance to multiple drugs, including those that are critically important for treating serious bacterial infections.

In the current article, WMS was also employed to assess the potential role of poultry products and poultry processing lines in the dissemination of ARGs, a field that is not yet extensively studied. The different poultry products collected in this study exhibited low abundances of ARGs, being consistent with findings from other WMS studies on poultry (Li et al., 2020) and beef products (Noyes et al., 2016). These results could be attributed to the interventions implemented during slaughtering and meat processing, which may effectively mitigate the risk of ARGs transmission to consumers (Noyes et al., 2016). Similarly, low ARG abundances were also observed in utilized surface samples, consistent with another study in which surfaces in meat industries showed a lower abundance of ARGs compared to surfaces in slaughterhouses (Alvarez-Molina et al., 2023). This observation suggested either that the introduction of ARGs through carcasses in meat industries is lower than the introduction of ARGs through animals in slaughterhouses, or that the C&D procedures were efficient in reducing microorganisms carrying ARGs. However, we detected different beta-lactams ARGs in clean surface samples, indicating that C&D procedures failed to remove all antimicrobial resistant bacteria. As those beta-lactams ARGs were strongly associated with different enterobacteria genera, the resistome of viable enterobacteria was comprehensively characterized through the sequencing of the DNA extracted from *Enterobacterales* plates scrapes. This in-depth analysis allowed the detection of these ARGs in different meat, utilized surfaces, burger, and expired burger samples, suggesting that clean surfaces might act as a reservoir of ARGs, which could be transferred to food products, potentially contributing to the spread of antibiotic resistance through the food supply chain.

Biocide resistance genes (*qacE* and *qacL*) were detected in both meat and utilized surface samples. The presence of these genes could be attributed to different causes. On the one hand, the inefficient implementation of C&D procedures (erroneous formulation, inappropriate storage, inadequate distribution, non-rotation of C&D agents, or application in the presence of organic matter or to wet surfaces) may expose bacteria to sub-inhibitory concentrations of C&D compounds, favoring the selection of bacteria with resistance to biocides (Alvarez-Molina et al., 2023). On the other hand, biofilm formation could serve as an adaptive response of bacteria to environmental stress (De Filippis et al., 2021), including the exposure to biocides during C&D procedures (Valentino et al., 2022). In the current study, the presence of these ARGs was strongly associated with the presence of *Pseudomonas* spp, a genus that has exhibited a robust capacity to form biofilms (Liu et al., 2015; Mascák et al., 2014). Biofilms are complex and structured cell communities embedded within a self-produced extracellular matrix of polymeric substances (López et al., 2010). This matrix limits the penetration of biocides into deeper regions of the biofilm, thereby exposing cells to sub-inhibitory concentrations and promoting the emergence and selection of biocides resistance (Flores-Vargas et al., 2021). Indeed, it has been demonstrated that the exposure to sub-inhibitory concentrations of quaternary ammonium compounds (QAC) – one of the most used biocides in the food industry – might enhance the acquisition of resistance to different drug classes through co/cross resistance (Merchel Piovesan Pereira et al., 2021; Nasr et al., 2018). In this sense, although the abundance of ARGs conferring resistance to biocides was not high, the so-called "disinfectant induced antibiotic resistance" (Chen et al., 2021) could adversely impact the health of the consumer (Jin et al., 2020).

Besides the presence of ARGs conferring resistance to biocides, the detection of high-risk ARGs in different samples throughout the burger processing line is worrying as they could pose serious threats to public health, especially if they confer resistance to last-resort antibiotics. An illustrative example was the *mcr*-4.2 ARG, which confers resistance to colistin – a crucial last-resort antibiotic reserved for combating human infections caused by multidrug-resistant Gram-negative bacteria (EFSA and ECDC, 2023).

5. Conclusion

To the best of our knowledge, this is the first study in which a combination of culturing techniques and WMS has been employed to assess the microbiome and resistome of a poultry burger processing line.

The microbiome analysis, employing both culturing techniques and WMS, provided us with a comprehensive understanding of the microbiota along the entire poultry burger processing line. This approach allowed for the identification of particular genera responsible for limiting the shelf-life of poultry burgers, as well as the genera that persists despite C&D procedures. The resistome analysis by WMS revealed the frequent occurrence of ARGs conferring resistance to biocides in different samples throughout the processing line, as well as the existence of antimicrobial resistant bacteria in surface samples after C&D procedures. All this information proves invaluable for designing novel food preservation processes and efficient C&D procedures targeted at combatting these persistent bacteria.

CRediT authorship contribution statement

Natalia Merino: Writing – original draft, Investigation, Data curation, Conceptualization. **Elisa Pagán:** Investigation. **Daniel Berdejo:** Writing – review & editing, Investigation, Conceptualization. **Colin J. Worby:** Writing – review & editing, Data curation, Conceptualization. **Mark Young:** Writing – review & editing, Conceptualization. **Abigail L. Manson:** Writing – review & editing, Conceptualization. **Rafael Pagán:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Ashlee M. Earl:** Writing – review & editing, Supervision, Conceptualization. **Diego García-Gonzalo:** Writing – review & editing, 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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114842.

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