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Microbiome dynamics, antibiotic resistance gene patterns and spoilage-associated genomic potential in fresh anchovies stored in different conditions

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ABSTRACT

Fresh fish is a highly perishable product and is easily spoiled by microbiological activity and chemical oxidation of lipids. However, microbial spoilage is the main factor linked with the rapid fish sensorial degradation due to the action of specific spoilage organisms (SSOs) that have the ability to dominate over other microorganisms and produce metabolites responsible for off-flavours.

We explored the microbial dynamics in fresh anchovies stored in different packaging (air, modified atmosphere, under vacuum) and temperatures (0, 4 and 10 °C) using shotgun metagenomics, highlighting the selection of different microbial species according to the packaging type. Indeed, *Pseudoalteromonas nigrifaciens*, *Psychrobacter cryohalolentis* and *Ps. immobilis*, *Pseudomonas deceptionensis* and *Vibrio splendidus* have been identified as the main SSOs in aerobically stored anchovies, while *Shewanella baltica*, *Photobacterium iliopiscarium*, *Ps. cryohalolentis* and *Ps. immobilis* prevailed in VP and MAP. In addition, we identified the presence of spoilage-associated genes, leading to the potential production of biogenic amines and different off-flavors (H_2S, TMA). In particular, the abundance of microbial genes leading to BA biosynthesis increased at higher storage temperature, while those related to H_2S and TMA production were enriched in aerobically and VP packed anchovies, suggesting that MAP could be an effective strategy in delaying the production of these compounds. Finally, we provided evidence of the presence of a wide range of antibiotic resistance genes conferring resistance to different classes of antibiotic (β-lactams, tetracyclines, polymyxins, trimethoprims and phenicols) and highlighted that storage at higher temperature (4 and 10 °C) boosted the abundance of ARG-carrying taxa, especially in aerobically and MAP packed fish.

1. Introduction

Seafoods are among the most popular and healthiest foodstuffs worldwide, containing a variety of essential elements for human diet such as proteins, vitamins, nutrients and long-chain polyunsaturated fatty acids, including omega-3 (Odeyemi et al., 2018; Anagnostopoulos et al., 2022).

It is generally accepted that the muscle tissue of healthy, live fish is sterile, and the natural microbiota occurs mainly in the slime layer on the skin, on the gills, and in the gastrointestinal tract. The total microbial loads usually change according to seasonal variations and depends on the pollution and temperature of the environment, on the method of catching and on the conditions of handling and storage. Due to the specific chemical composition, such as a high content of nutrients, especially non-protein nitrogenous compounds (NPN), high water activity (a_w) and a pH close to the neutrality, seafood shelf-life is particularly short, and it represents an optimal substrate for the growth of different spoiling and pathogenic microbes (Sequino et al., 2022).

Spoilage is mainly the result of the metabolic activity of a fraction of the initial microbiota, the so-called Specific Spoilage Organisms (SSOs), which are responsible for the degradation nutrients, leading to the production of off-flavors and to a loss of the texture, making the product unacceptable for the consumption. Poor hygiene or sanitary practices and improper conditions in harvesting, handling, storage, processing,

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and distribution, strongly affect the microbiota profile and activity in seafood, promoting the growth of SSOs, and influencing both the rate and course of spoilage (Gram & Dalgaard, 2002; Anagnostopoulos et al., 2022).

Proteins, especially structural proteins, are hydrolyzed by microbial proteases into peptides and amino acids, resulting in changes in the physicochemical properties (texture, moisture distribution, color, water holding capacity, etc.) of fish flesh. Amino acids undergo transamination, deamination and decarboxylation leading to the production of α-keto acids, ammonia, trimethylamine and various kinds of biogenic amines (Zhuang et al., 2021; Bulushi et al., 2009). Trimethylamine (TMA) content, total volatile bases (TVB), individual nucleotides and nucleotide ratios (K, Ki, H and G-values) have been proposed as indices of deterioration of fish quality. Levels of biogenic amines can also be useful in estimating the fish freshness since their formation is associated with bacterial spoilage (Özogul et al., 2004).

In addition, microbial decomposition of sulfur-containing, branched-chain and aromatic amino acids have attracted attentions because several volatile organic compounds (VOCs) with off-odor are synthetized by their catabolism. Finally, visible slime may also appear as a result of the production of extracellular polysaccharides (Sequino et al., 2022; Biji et al., 2016).

The different storage and packaging conditions (atmosphere, storage temperature and the application of antibacterial compounds) may have a major effect on microbial growth and dynamics of different populations (Masniyom, 2011). Modified atmosphere packaging (MAP) and vacuum-packaging (VP), along with refrigeration, have become increasingly popular preservation techniques, which have brought major changes in storage, distribution and marketing of raw and processed products to meet consumer demands. MAP and VP systems may help in improving seafood shelf life, organoleptic quality and product range (Özogul et al., 2004).

Previous investigations have shown that psychrotrophic members of the genera *Pseudomonas*, *Shewanella* and *Psychrobacter* prevail in chilled fish stored in aerobic conditions (Zotta et al., 2019). However, these genera may be replaced by *Photobacterium*, *Brochothrix* and lactic acid bacteria (LAB) in products stored in MAP or VP (Silbande et al., 2018).

Moreover, multiple studies have shown that some pathogenic/commensal bacteria associated with food and its production environment may carry out Antibiotic Resistance Genes (ARGs) in their genomes (Oniciuc et al., 2019; Valentino et al., 2022). According to WHO, antibiotic resistance (AR) is one of the most important public concerns, since the overuse of antibiotics in all fields (e.g., agriculture, farming and individual medications) has led to the selection of resistant strains (Ventola, 2015; World Health Organization, 2020). The increasing numbers of reports on the presence of antibiotic resistance in food bacteria are indicative of an important public health concern and, consequently, there is an urgent need to limit the spread of ARG within food chains, since they may be transferred to opportunistic and pathogenic bacteria.

In this work, we used a metagenomic approach to assess the influence of different packaging technology and storage temperature on the microbiome dynamics and functional potential during fresh anchovies shelf life and to evaluate their influence on the pattern of ARGs. 'Omics may be successfully implemented within the food industry, assessing microbiome dynamics and activities during food shelf life, the impact of different processing technologies and storage conditions, and assisting in predicting the shelf life. In addition, the integration of this approach into risk assessment tools may help in identifying the presence of potential food safety concerns and the possible contamination routes.

2. Materials and methods

2.1. Samples collection and packaging preparation

Fresh anchovies were bought in a fish market located in Campania region (Southern Italy). The head and visceral were removed at the moment of purchase. Two different samplings were carried out in December 2021 and January 2022. They were transported to the laboratory in ice within 30 min, and then packed in trays with an absorbent sheet to avoid excessive accumulation of exudates. Three packaging conditions were used:

Air packaging (AIR): anchovies were heat-sealed under air, with polystyrene tray laminated with a multilayer barrier film (V = 500 cc; CoopBox, Bologna, Italy) and sealed with a film of PA/EVOH/PE (PO_2 = 1.3 cm^3 m^{-2} 24 h^{-1} atm^{-1} at 23 °C, 0 % RH) by using a packaging machine (TSM 105 Minipack-Torre, Dalmine, Bergamo, Italy). On the bottom of the tray was placed an absorbent layer to avoid excessive accumulation of exudates The ratio between the volume of gas and weight of food product (G/P ratio) was 4:1 (V/W).

Modified atmosphere packaging (MAP): anchovies were packed were heat-sealed by using the same try, film and packaging machine of air packed samples, but under modified atmosphere packaging (MAP) with 20 kPa O2 and 60 kPa CO_2 (Torrieri et al., 2006).

Vacuum packaging (VP): anchovies were placed in 23 x 14 cm polystyrene trays and vacuum packed in LDPE channeled bags (30x20 cm) by using the vacuum packaging machine (Lavezzini Model Jolly new gas, Fiorenzuola D'Arda, Piacenza, Italy).

All samples were stored at three different temperatures (0, 4, 10 °C) and analysed at different time points. We selected these temperatures to simulate the normal storage conditions of fresh fish, as well as a temperature abuse. Samples at 0 and 4 °C were stored up to 10 days (aerobically) and 16 days (MAP and VP). Samples at 10 °C were stored up to 7 days in all packaging conditions, since the deterioration was evident after this period.

For air and modified atmosphere packaging conditions, the headspace gas composition was monitored using a O_2/CO_2 gas analyzer (accuracy of 0.5 %), equipped with a needle (Check Mate 9900 O2/CO2; Ringsted, Denmark) during storage at 0 and 10 °C (Table S1).

2.2. DNA extraction and whole metagenome sequencing

Samples were weighted (~30 g) and transferred to a sterile bag, where STE (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) buffer was added in 5:1 ratio and microorganisms were detached from the surface of the anchovies by shaking, without damaging the tissues and limiting the release of fish epithelial cells. About 100 mL of STE solution containing the microorganisms were collected and centrifuged at 12,000 × *g* for 2 min, then the pellet was washed twice with 2 mL of sterile STE and stored at − 80 °C until DNA extraction. DNA extraction was performed from the pellets using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and quantified using the Qubit HS Assay (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

Metagenomic libraries were prepared using the Nextera XT Index Kit v2 (Illumina, San Diego, California, United States), then whole metagenome sequencing was performed on an Illumina NovaSeq platform, leading to 2 × 150 bp, paired-end reads.

2.3. Bioinformatic analysis

Host reads contamination was removed mapping reads to the *Sardina pilchardus* (*European pilchard*) genome (NCBI Accession Number: PRJNA481265) by using the Best Match Tagger (BMTagger; https://www.hmpdacc.org/hmp/doc/HumanSequenceRemoval_SOP.pdf).

The resulting reads were quality-checked and filtered through Prinseq-lite v. 0.20.4, using parameters '-trim_qual_right 5′ and '-min_len 60′ (Schmieder & Edwards, 2011). Taxonomic and functional profiles were obtained through MetaPhlAn v. 4.0 and HUMAnN v. 3.6, respectively (Blanco-Míguez et al., 2023; Beghini et al., 2021). Genes/pathways from HUMAnN outputs were relabeled according to the UniRef90

annotations.

For each sample, high-quality reads were assembled into contigs using MEGAHIT v. 1.2.2 (Li et al., 2015), filtering out contigs < 1,000 bp. Reads from each sample were mapped to the corresponding contigs using Bowtie2 v. 2.2.9 (Langmead & Salzberg, 2012), with parameters '–very-sensitive-local' and '-–no-unal'.

Furthermore, filtered reads were screened for AR genes, through mapping against the ResFinder database (Florensa et al., 2022); https://bitbucket.org/genomicepidemiology/resfinder_db/downloads/) using Bowtie2 v. 2.2.9 (option: '–very-sensitive-local'; Langmead & Salzberg, 2012). Genes were grouped according to the antibiotic class they confer resistance to. Those genes conferring resistance to macrolides, lincosamides, streptogramins and pleuromutilins were collapsed into the MLSP class, while those that confer resistance to oxazolidinones into the oxazolidinone class as previously reported (Additional file 9 - Phenotypes table from Cobo-Díaz et al., 2021). Abundance matrices were transformed to count per million reads (CPM) matrices for further analyses using an R-script (https://github.com/JoseCoboDiaz/counts2CPM).

2.4. Statistical analysis

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

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

Taxa correlations were computed with 'cor.test' function from the 'psych' package and the FDR method was used to correct p-values, whereas the plot was obtained by 'corrplot' function from the 'corrplot' package. The linear regression analysis between taxa and genes involved in biogenic amines production was visualized by the function 'ggscatter' from the 'ggpubr' package using the Spearman method to calculate correlation coefficients (R) and p-values.

Barplot and heat plot were produced by the functions 'barplot' and 'pheatmap' from the 'base' and 'pheatmap' packages, respectively. Finally, the function 'geom_line' from the 'ggplot2′ package was used to make the line plot, showing the most abundant ARGs for each group.

2.5. Data availability

The raw sequence reads generated in this study have been deposited in the Sequence Read Archive (SRA) of the National Center of Biotechnology Information (NCBI) under the accession number PRJNA1034056.

3. Results

3.1. Packaging and storage temperature shape the taxonomic composition and functional patterns of the microbiome during fresh anchovies storage

Different microbiome composition was found in samples at baseline collected during different samplings. Anchovies from the second sampling showed a greater microbial diversity (Fig. 1). While *Photobacterium aquimaris* dominated in all baseline samples (average 97.90 % and 57.64 %, in the first and second experiment, respectively), *Brochothrix thermosphacta* and several species belonging to *Shewanella* and *Psychrobacter* spp. were present only in baseline anchovies from the second sampling. Consistently, anchovies from the second sampling maintained higher microbial diversity during the shelf life, regardless the temperature and the packaging conditions (Fig. 2A). During the storage, *Brochothrix thermosphacta*, and several species of *Shewanella* and *Psychrobacter* increased in abundance, although a more complex microbiome persisted (Fig. 2A). Conversely, *Photobacterium aquimaris* was dominant at baseline in anchovies belonging to the first sampling, but during the shelf life, its abundance strongly decreased, and it was replaced by other *Photobacterium species (Ph. iliopiscarium and Ph. toruni), Shewanella baltica* and *Sh. frigidimarina* (Fig. 2A).

Considering the different packaging conditions, *Pseudoalteromonas nigrifaciens, Ps. cryohalolentis* and *Ps. immobilis, Pseudomonas deceptionensis* and *Vibrio splendidus* were more abundant during storage in AIR packaging. Samples packed in VP and MAP showed similar profiles, with *Sh. baltica, Photobacterium iliopiscarium, Ps. cryohalolentis* and *Ps. immobilis* identified as main SSOs, although in several cases these taxa were more abundant in VP compared with MAP (Fig. 2B).

In order to investigate the ecological relationship between microbial taxa, we looked at specific co-occurrence and co-exclusion patterns. We observed that *Photobacterium aquimaris* co-excluded with several other species (e.g., *Pseudomonas deceptionensis*, *Brochothrix thermosphacta*, *Carnobacterium*, several species of *Psychrobacter* and *Shewanella*), highlighting possible antagonistic dynamics among these taxa, whereas *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter* and *Shewanella* spp. significantly co-occurred (FDR corrected *p*-value < 0.05; Fig. 2C).

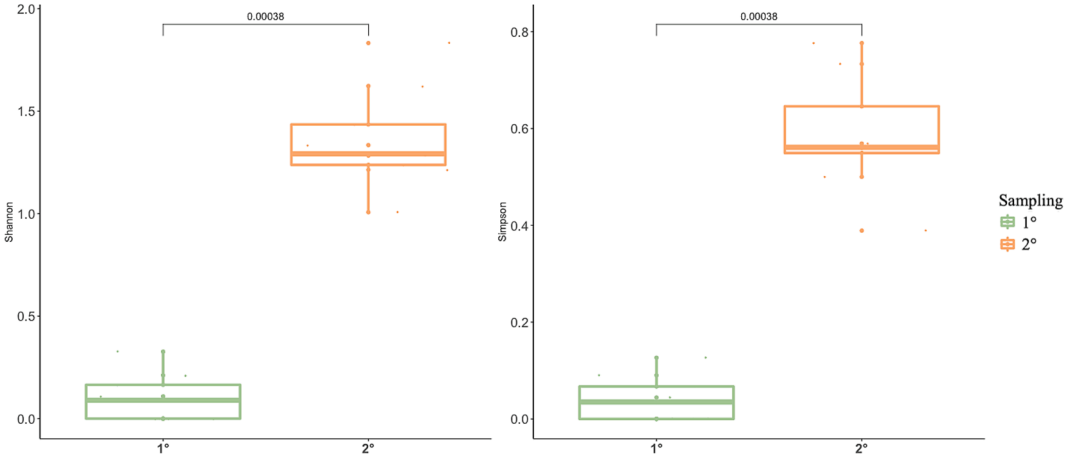

Fig. 1. Box plots showing the Shannon and Simpson diversity indices at baseline of anchovies belonging to the first and second sampling. P-values were calculated using Wilcoxon Rank Sum Tests. Green: First sampling; Orange: Second sampling. Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.5 IQR from the first and third quartiles, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

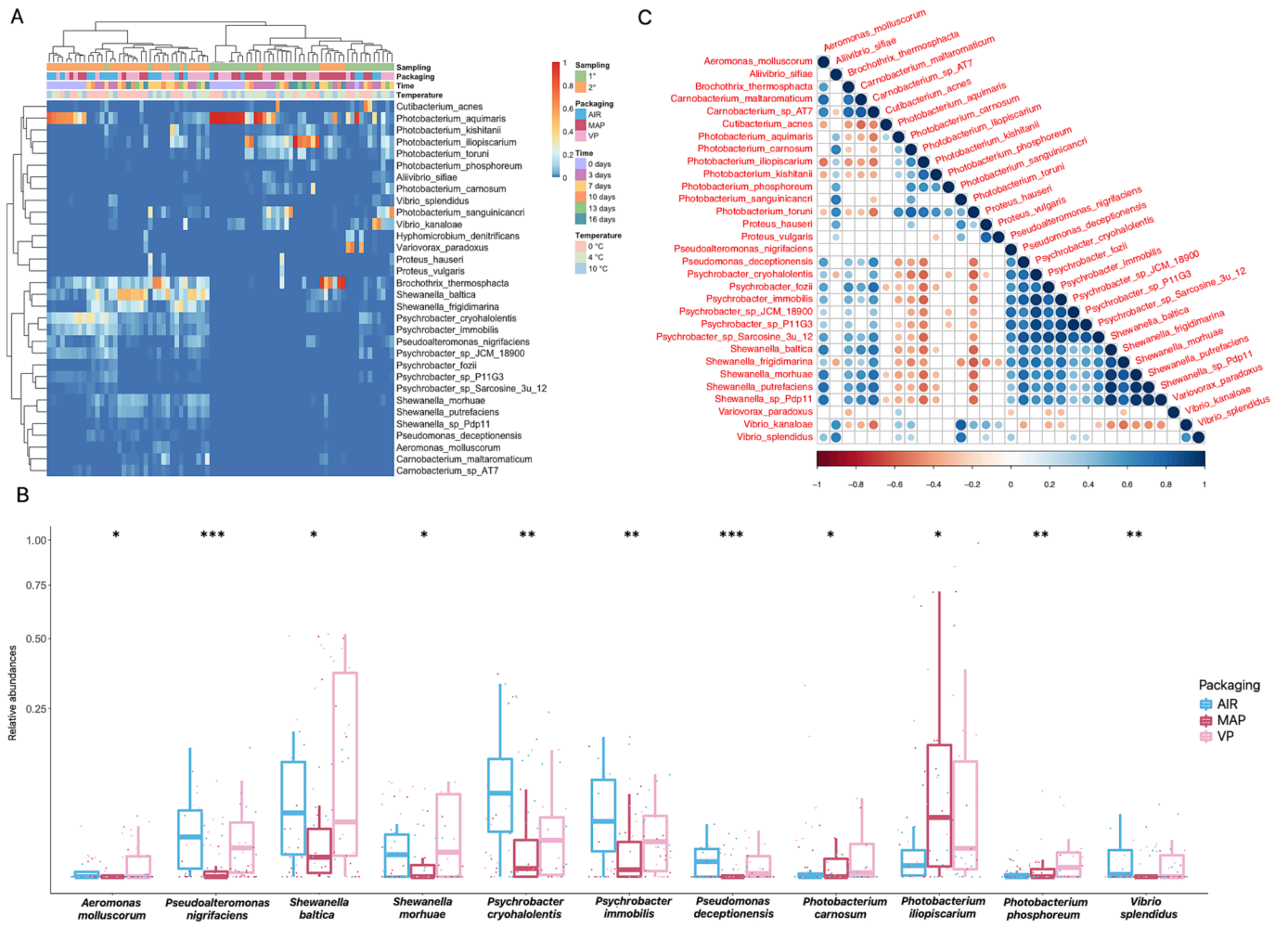

Fig. 2. A: Heat plot showing the abundance (%) of the most abundant microbial taxa in the fish samples analyzed. Only taxa with abundances > 1 % are included. The column bars are color-coded according to the sampling, the packaging conditions, the different time points and the storage temperature. B: Box plots showing the relative abundances of dominant fish spoilage bacteria in the different packaging conditions. P-values were calculated using Kruskal–Wallis Tests. Significance codes: '***' p-value between 0 and 0.001, '**' p-value between 0.001 and 0.01, '*' p-value between 0.01 and 0.05. C: Spearman's rank correlations between microbial taxa. Taxa with abundance > 1 % are considered. The intensity of the colors and the size of the circles represent the degree of association as measured by Spearman's correlations. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and − 1 indicating a perfectly negative correlation (dark red) between two microbial taxa. Only significant correlations (FDR < 0.05) are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We also investigated the functional potential of the microbiome. In fresh fish, biogenic amines (BAs) are produced as a consequence of the decarboxylation of the free precursor amino acids, through the action of substrate-specific microbial decarboxylases and are considered as a marker of freshness. Genes coding for histidine, tyrosine, lysine and arginine decarboxylases were found in all the samples and their abundance was enriched in samples stored at 4 and 10 °C compared with 0 °C (Fig. 3A). Furthermore, several genes coding for hydrogen sulfide (H_2S) and trimethylamine (TMA) production, responsible for putrid and ammonia-like off-flavours, respectively, were found in all the samples but at higher abundance in anchovies stored in AIR (Fig. 3B).

In addition, a linear regression model demonstrated a strong positive correlation between the relative abundances of some taxa and genes involved in biogenic amines production. In particular, *Shewanella baltica*, *Psychrobacter immobilis*, *Photobacterium iliopiscarium* and *Shewanella morhuae* were positively correlated with agmatine deiminase, ornithine decarboxylase and arginine decarboxylase, microbial genes involved in the biosynthesis agmatine and putrescine (Fig. 4).

3.2. Anchovies in different packaging and storage conditions host a broad range of ARGs

Reads were screened for the presence of ARGs, highlighting an increase in the total amount of ARGs (although not statistically significant) over time in the different packaging conditions (Supplementary Figure 1A) and storage temperature (Supplementary Figure 1B).

The most abundant ARGs were associated with resistance to β-lactams, that together with polymyxins and tetracyclines were the most abundant AR classes. Storage at higher temperature (4 and 10 °C) boosted the abundance of ARG-carrying taxa in aerobic and MAP packaging, while the highest abundance of ARGs was detected in anchovies packed in VP and stored at 0 °C (Fig. 5).

Besides β-lactams, genes related with tetracyclines resistance were rated second in terms of abundance, in particular in anchovies stored in AIR at 10 °C, in VP at 4 °C and in MAP both at 4 and 10 °C. Polymyxins resistance genes, on the other hand, were particularly abundant in VP samples. Finally, trimethoprims- were more abundant in aerobically stored anchovies at 4 and 10 °C, and phenicols-resistance genes were detected only at 10 °C in air pack (Fig. 5).

Several different determinants of resistance to tetracyclines (*tet(M)*,

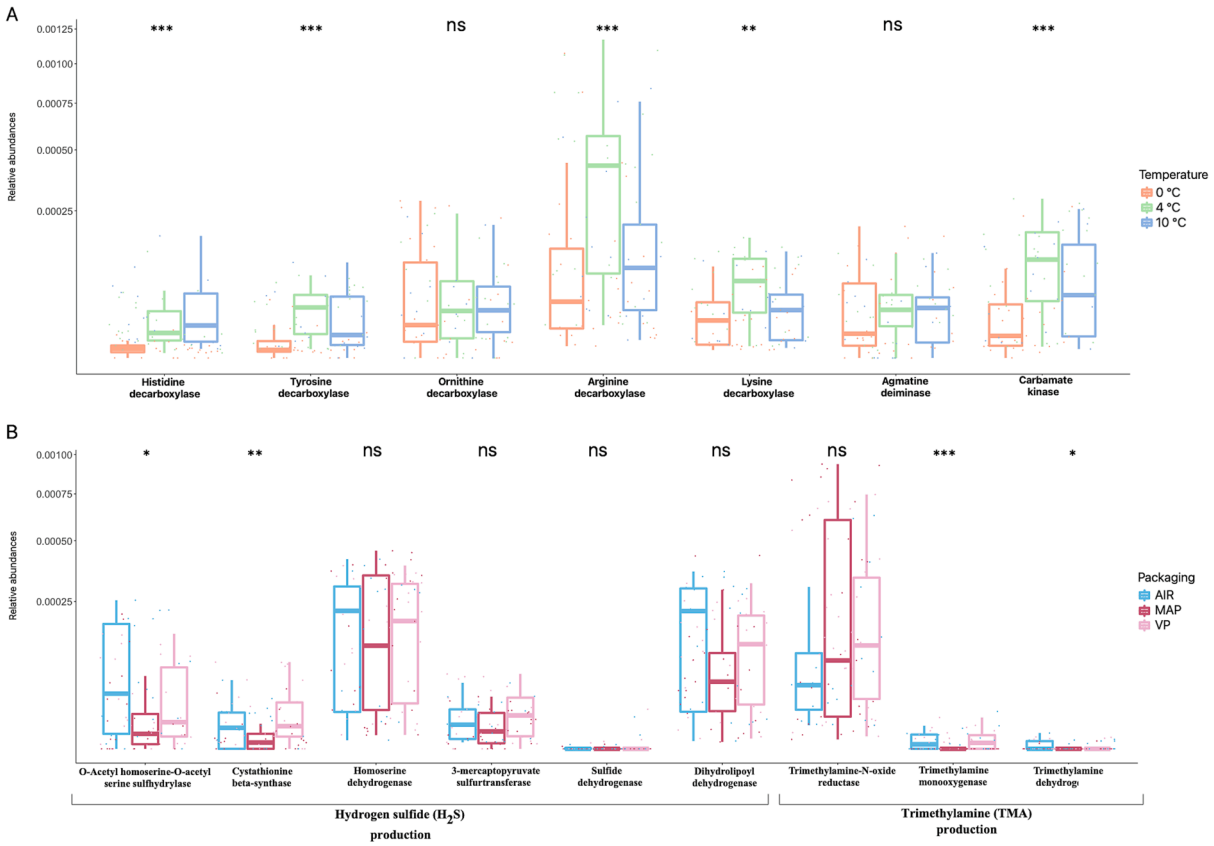

Fig. 3. Box plots showing the relative abundances of genes involved in BA production in samples grouped according to the different storage temperature (A) and of genes involved in H_2S and TMA production in samples grouped according to the type of packaging (B). P-values were calculated using Kruskal–Wallis Tests. Significance codes: '***' p-value between 0 and 0.001, '**' p-value between 0.001 and 0.01, '*' p-value between 0.01 and 0.05, 'ns' not significant.

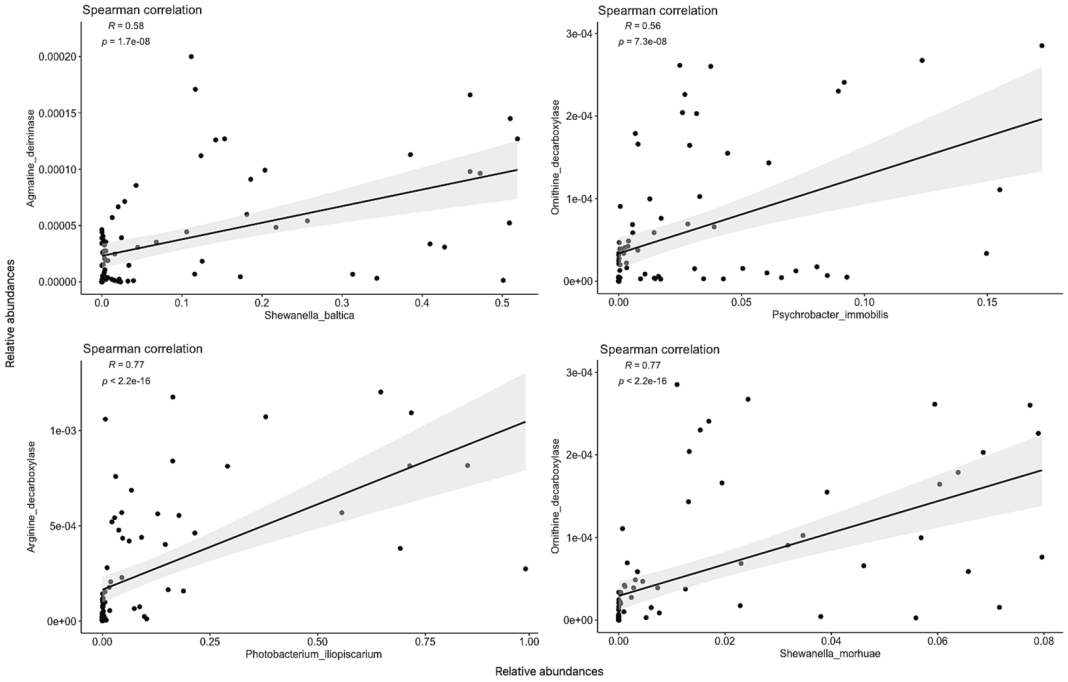

Fig. 4. Spearman's correlation between the relative abundances of taxa and genes involved in biogenic amines production.

tet(L), *tet(H)*, *tet(E)*, *tet(D)*), sulfunamides (*sul1*), polymyxins (*mcr-4.6*, *mcr-4.5*, *mcr-4.4*, *mcr-4.3*, *mcr-4.2*, *mcr-4.1*), β-lactams (*hugA*, *cphA5*, *cphA1*, $bla_{OXA-553}$, $bla_{OXA-552}$, $bla_{OXA-550}$, $bla_{OXA-549}$, $bla_{OXA-548}$, bla_{DHA-16}), trimethoprims (*dfrA6*), phenicols (*catA2*) and aminoglycosides (*ant(3'')-Ia*) were among the most abundant ARGs found in anchovies microbiome (Fig. 6).

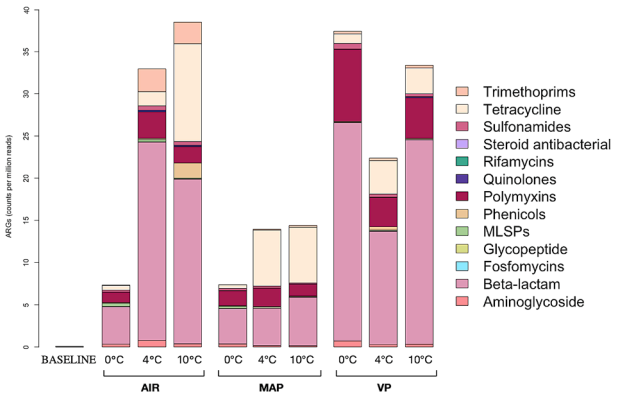

Fig. 5. Barplot showing the abundance in counts per million reads (CPM) of the Antibiotic Resistance Genes classes. Baseline indicates all samples at time 0, regardless of packaging and temperature conditions. Each bar represents the average value for samples belonging to the two replicate experiments. MLSPs refers to the sum of macrolides, lincosamides, streptogramins and pleuromutilins classes.

Mcr-4.6, *mcr-4.5*, *mcr-4.4*, *mcr-4.3*, *mcr-4.2*, *mcr-4.1* genes, associated with resistance to polymyxins, and $bla_{DHA\text{-}16}$ gene, associated with resistance to β-lactams, were the most abundant determinants associated with resistance to Critically Important Antibiotics (CIA), as described by the World Health Organization (World Health Organization, 2018; EFSA BIOHAZ Panel et al., 2021). In particular, the microbiome in VP anchovies showed higher diversity of *mcr* and *bla* genes, although the total counts were lower compared with AIR and MAP samples (Fig. 6).

4. Discussion

It is well known that changes in microbiota composition can have important impacts on the spoilage patterns of fresh fish (Zhuang et al., 2021). Generally, the microbiota composition of fresh anchovies at baseline reflects that of the surrounding water (Feldhusen, 2000) which is dominated by psychrotrophic, Gram-negative, rod-shaped bacteria belonging to the genera *Photobacterium*, *Shewanella*, *Psychrobacter*, *Vibrio* and *Pseudoalteromonas*, consistently with previous reports. *Shewanella putrefaciens* and *Shewanella baltica* are common dominant spoilage bacteria in iced sea salmon (Hozbor et al., 2006; Macé et al., 2012), gutted sea bass (Parlapani et al., 2015), chilled fresh Mediterranean swordfish (Pantazi et al., 2008), tropical prawns (Chinivasagam et al., 1998; Zhu et al., 2015) and large yellow croaker (Zhu et al., 2016), whereas *Pseudoalteromonas* and *Vibrio* spp. have been previously reported in oysters during iced storage (Madigan et al., 2014). In addition, *Psychrobacter* and *Pseudoalteromonas* spp. were detected as main spoilers in cooked brown shrimp, where they are involved in lipid and protein hydrolysis (Broekaert et al., 2013b). It is noteworthy that Photobacterium phosphoreum is known to be a psychrotrophic and halophilic histamine producer, which is common in the marine environment and has been shown to be the dominant histamine producer when fish is stored at temperatures < 15 °C (Lehane & Olley, 2000; Kanki et al., 2004).

Consistently we identified that temperature and packaging type can strongly affect fresh fish microbiome during storage. Understanding how these factors may shape the functional potential of the microbiome, may support the implementation of appropriate methods for ensuring the quality and safety of fishery products and extending their shelf life. Indeed, the microbiome during the shelf life was dominated by *Psychrobacter*, *Pseudoalteromonas* and *Shewanella* spp., previously identified as SSOs in aerobically stored fish (Broekaert et al., 2013a; Zhuang et al., 2021; Gram & Dalgaard, 2002), while *Shewanella* and *Photobacterium* spp. were more associated with VP and MAP, as reported by other authors (Hoel et al., 2022; Hansen et al., 2021; Kuuliala et al., 2018). However, *Shewanella* spp. were identified as main SSOs in all packaging conditions. Some of these taxa have been reported as able to reduce trimethylamine N-oxide (TMAO), producing trimethylamine (TMA), that contributes to the characteristic ammonia-like and 'fishy' off-flavours (Gram, 2009; Summers et al., 2017; Gram & Dalgaard, 2002). *Shewanella* spp. can also release hydrogen sulfide (H_2S) by degrading the sulfur-containing amino acids, which result in putrid off-odors in various fish products (Remenant et al., 2015; Vogel et al., 2005; Teymouri & Shekarchizadeh, 2022). We identified the genes potentially involved in these pathways in all the samples, with higher levels in AIR and VP stored samples, suggesting that MAP (high concentration of carbon dioxide and aerobic condition, Table S1) may be more effective in delaying the production of these compounds. However, we have to point out that also in AIR packaging, O2 was quicky consumed during storage, most of all at high temperature, creating an anoxic condition (Table S1).

Our results also demonstrated a strong positive correlation between the relative abundance of several taxa and genes involved in BAs production. In particular, *Shewanella*, *Psychrobacter* and *Photobacterium* were the taxa showing the highest correlation, suggesting their important role in BA production, as reported previously (Lan et al., 2021; Zhu et al., 2016; Bjornsdottir-Butler et al., 2018). Although BA concentration was not measured in this study, we observed that the abundance of microbial genes leading to BA biosynthesis increased as a function of the storage temperature, highlighting that it is an important factor contributing to BA formation. The consumption of seafoods containing high levels of BAs may result in severe intoxication (Hu et al., 2012).

Although we did not carry out chemical and sensorial analyses, our results may pose the bases for further studies confirming the effects of microbial activities on sensorial profiles.

Moreover, we identified several correlations among members of the microbiome, that may suggest some ecological interactions. Microbial interactions are known to affect spoilage and fermentation of fish products (Joffraud et al., 2006; Jørgensen et al., 2000; Macé et al., 2013) and may provide useful insights in the factors that shape the composition of microbial communities (Parente et al., 2018). Interestingly, *Photobacterium aquimaris* co-excluded with several other taxa (e.g., *Pseudomonas deceptionensis*, *Brochothrix thermosphacta*, *Carnobacterium*, *Psychrobacter* and *Shewanella* spp.), highlighting that this species dominates the fresh fish microbiota, as previously reported (Alfaro & Hernandez, 2013). However, in the late spoilage stages, its abundance strongly decreases, and it is replaced by other *Photobacterium* species (*Ph. iliopiscarium* and *Ph. toruni*), *Shewanella baltica* and *Sh. frigidimarina*. On the contrary, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter* and *Shewanella* spp. co-occurred. Indeed, *Pseudoalteromonas* has a large biochemical potential and may create extra-nutrients for the growth and metabolic activities of the other taxa. In particular, it was suggested to be able to enhance the spoilage activity of *Psychrobacter* during brown shrimp storage (Broekaert et al., 2013b).

Although the mechanisms underlying such patterns are not clear, these data my pose the bases for further efforts in deciphering interactions among bacteria on fish products, that might be useful to adopt specific and focused biocontrol or preservative procedures to improve safety and extend the shelf life.

Moreover, we identified the presence of a wide variety of AR genes in fish microbiome, mainly associated with resistance to β-lactams, tetracyclines, polymyxins, trimethoprims and phenicols antibiotic classes, consistently with what has been previously reported (Ferri et al., 2022; Mo et al., 2017; Amarasiri et al., 2020). Specifically, several different determinants conferring resistance to tetracyclines (*tet(M)*, *tet(L)*, *tet(H)*, *tet(E)*, *tet(D)*), polymyxins (*mcr-4.6*, *mcr-4.5*, *mcr-4.4*, *mcr-4.3*, *mcr-4.2*, *mcr-4.1*) and β-lactams (*hugA*, *cphA5*, *cphA1*, $bla_{OXA\text{-}553}$, $bla_{OXA\text{-}552}$, $bla_{OXA\text{-}550}$, $bla_{OXA\text{-}549}$, $bla_{OXA\text{-}548}$, $bla_{DHA\text{-}16}$) were among the most abundant ARGs found in anchovies microbiome. Several hot spots of AMR genes have been identified in water bodies, such as seawater, oceans, and fresh water (Cuadrat et al., 2020; Schar et al., 2021; Singh et al.,

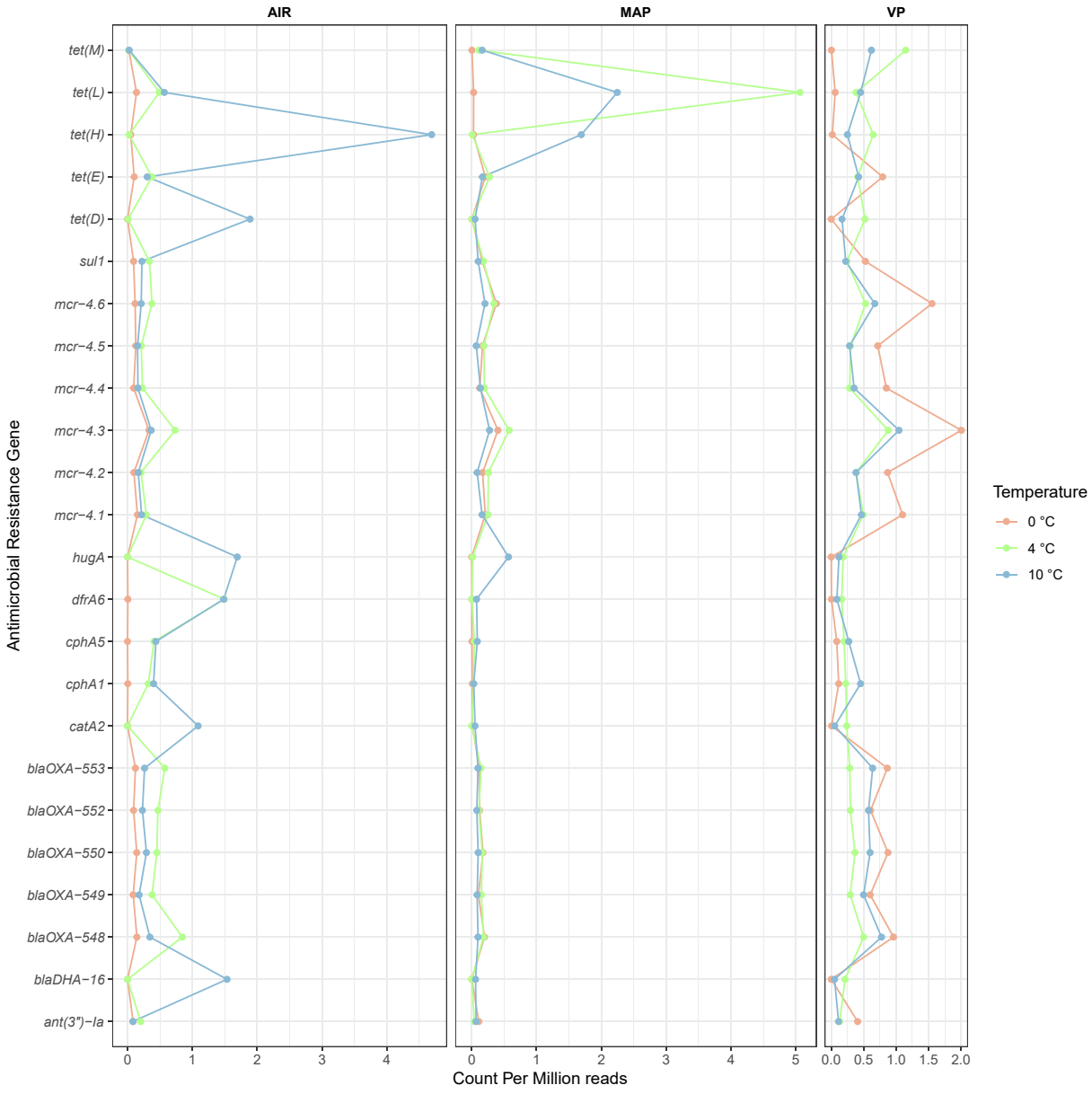

Fig. 6. Line plot that shows the most abundant antimicrobial resistance genes in counts per million reads (CPM) found in anchovies microbiome during storage.

2022), and including the Mediterranean Sea (Gambino et al., 2022), where the anchovies analysed in this study were caught. Although higher risk is associated with aquaculture and reared fish, due to the direct use of antibiotics during rearing (Obayashi et al., 2020; Higuera-Llantén et al., 2018; Muziasari et al., 2017; Domínguez et al., 2019), our results confirmed the presence of a wide AMR pattern also in marine fish microbiome. In particular, genes conferring resistance to polymyxin, β-lactam and tetracycline were the most abundant. Interestingly, previous report identified these AMR classes as the most common in marine bacteria (Hatosy & Martiny, 2015; Gambino et al., 2022) and AMR genes are often harboured on plasmids or other mobile elements that can be easily transferred to bacteria of different genera and kingdoms (Hamza et al., 2020; Lima et al., 2019).

5. Conclusion

Food spoilage leads to significant waste, and it is an important economic issue for food industry. Our results highlighted that different packaging and storage conditions affect fresh fish microbiome during shelf-life, as well as its functional potential, identifying several microbial genes potentially related to spoilage. By gaining a deeper understanding of these interactions, we can pave the way for more effective preservation methods, ensuring the freshness and safety of these products. The present work can be the baseline for further investigation, allowing these data to be integrated into models able to predict the nature and the speed of spoilage dynamics, based on the microbial community description at the beginning of the shelf-life, contributing to reduce food waste and to a more sustainable fishery industry. Although this type of fish usually has an extremely short shelf-life, the use of new approaches (e.g. combination of different packaging and low temperature) may help in extending the shelf-life, supporting the export of this local fish in foreign markets, as well as reducing the wastes.

CRediT authorship contribution statement

Giuseppina Sequino: Data curation, Formal analysis, Writing – original draft. **Vincenzo Valentino:** Formal analysis. **Alessia Esposito:** Formal analysis. **Stefania Volpe:** Formal analysis. **Elena Torrieri:**

Writing – review & editing. **Francesca De Filippis:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. **Danilo Ercolini:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data are available for download and accession number is provided in the text

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113788.

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