Shotgun metagenomic investigation of foodborne pathogens and antimicrobial resistance genes in artisanal fermented meat products from the Mediterranean area

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Abstract

In this pilot study, we compared the metagenomic profiles of different types of artisanal fermented meat products collected in Italy, Greece, Portugal, and Morocco to investigate their taxonomic profile, also in relation to the presence of foodborne pathogens and antimicrobial resistance genes. In addition, technical replicates of the same biological sample were tested to estimate the reproducibility of shotgun metagenomics. The taxonomic analysis showed a high level of variability between different fermented meat products at both the phylum and genus levels. *Staphylococcus aureus* was identified with the highest abundance in Italian fermented meat; *Escherichia coli* in fermented meat from Morocco; *Salmonella enterica* in fermented meat from Greece; *Klebsiella pneumoniae* and *Yersinia enterocolitica* in fermented meat from Portugal. The fungi *Aspergillus*, *Neosartoria*, *Emericella*, *Penicillum* and *Debaryomyces* showed a negative correlation with *Lactococcus*, *Enterococcus*, *Streptococcus*, *Leuconostoc* and *Lactobacillus*. The resistome analysis indicated that genes conferring resistance to aminoglycoside, macrolide, and tetracycline were widely spread in all samples. Our results showed that the reproducibility between technical replicates tested by shotgun metagenomic was very high under the same conditions of analysis (either DNA extraction, library preparation, sequencing analysis, and bioinformatic analysis), considering both the degree of overlapping and the pairwise correlation.

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Key words: shotgun metagenomic, fermented meat, biological hazards, antimicrobial resistance, Mediterranean productions.

Contributions: VI, carried out the bioinformatic and biostatistics analysis and writing of the original draft; CO, AL, performed DNA extraction, library preparation and sequencing; FS, contributed to the paper drafting and editing; UGB, PS, FA, collected and shipped the samples from their own country; GM, AS, AD, conceived and planned the study, collected the Italian samples and supported in editing the final draft. All authors have commented and approved the final version as submitted.

Conflict of interest: the authors declare no potential conflict of interest.

Ethics approval and consent to participate: not applicable.

Funding: this research was funded by the EU H2020 PRIMA ARTISANEFOOD project "Innovative Bio-interventions and Risk Modeling Approaches for Ensuring Microbial Safety and Quality of Mediterranean Artisanal Fermented Foods".

Availability of data and materials: data and materials are available from the corresponding author upon request.

Conference presentation: this paper was presented at the XXXII National Conference of the Italian Association of Veterinary Food Hygienists (AIVI), September 13-15, 2023, Maierato, Italy.

Received: 20 December 2023.
Accepted: 8 January 2024.
Early access: 10 May 2024.

Italian Journal of Food Safety 2024; 13:12210
doi:10.4081/ijfs.2024.12210

Introduction

The production of artisanal fermented meats, such as dry-cured sausages and salami, is part of the Mediterranean tradition and their consumption is widespread in many countries. Artisanal fermented meats are products in which the fermentation process contributes significantly to the unique organoleptic characteristics and flavors but also the microbiological safety.

The organoleptic features of artisanal fermented meats are the result of a complex interplay between raw materials, traditional craftsmanship, fermentation conditions, and microorganisms' presence that are typical of the production area. The metabolic activity of the microorganisms, as lipid and protein degradation, produces volatile compounds contributing to the characteristic aroma and flavor and influencing the product texture. On the other hand, the lack of standardized hygienic production practices can make artisanal fermented meat products more susceptible to contamination by harmful pathogens in comparison to more industrial products (Halagarda and Wójciak, 2022).

Shotgun metagenomic has been applied to provide a comprehensive and unbiased view of the microbial communities in food products, including fermented meat (Srinivas *et al*., 2022). This

sequencing methodology allows the simultaneous analysis of all DNA extracted from a sample, providing advantages in terms of taxonomic classification in comparison to culture-based methods, which only capture a limited fraction of the microbial community that is cultivable. Beyond the taxonomic profiling, shotgun metagenomic provides information about the functional genes of the microbial community, which is critical for understanding the ecosystem's capabilities and activities (Handelsman, 2004).

In comparison to traditional culture-based methods, the use of shotgun metagenomic for foodborne pathogen detection can facilitate the detection of outbreaks caused by non-culturable pathogens (or with unknown etiology) and is also suitable for identifying mixed infections. On the other hand, some methodological limitations, such as the lack of harmonized and standardized methods, detection sensitivity and specificity constraints, the high costs of the sequencing run and the time needed for the computational analysis still represent important challenges we need to overcome to extend the application of shotgun metagenomic in food inspection and for outbreak investigation (Koutsoumanis *et al*., 2019).

In this pilot study, we compared the metagenomic profiles of different types of artisanal fermented meat products collected in Italy, Greece, Portugal, and Morocco to investigate their taxonomic profile, also in relation to the presence of foodborne pathogens and antimicrobial resistance genes (ARGs). In addition, technical replicates of the same biological sample were tested to estimate the reproducibility of shotgun metagenomic.

Materials and Methods

Sample collection and processing

A total of 13 meat samples were collected from Italy, Morocco, Portugal, and Greece. The Italian samples were: 2 samples of pork meat mixture used as raw material for the salami production (*i.e.*, samples labeled as IT3 and IT4); 2 samples of salami cured for 1 and 3 weeks, respectively (*i.e.*, samples labeled as IT5 and IT6); 3 samples of salami cured for 18 weeks (*i.e.*, samples labeled as IT1a, IT1b; IT1c); 2 technical replicates of the samples IT1a and IT1b (*i.e.*, samples labeled as IT2a and IT2b). The Greek samples were 2 pork-based Noumboulo (*i.e.*, samples labeled as EL1 and EL2). The Portuguese sample was a pork and poultry-based Alheira de Mirandela (*i.e.*, sample labeled as PT1). The Moroccan sample was a sheep-based Merguez (*i.e.*, sample labeled as MA1). A total of 10 g of each sample were diluted in 90 mL of sterile NaCl 0.90%, homogenized using the stomacher (MAYO HG 400V, Italy) for 1 minute at normal speed, and centrifuged for 20 minutes at 9980× g at 4°C. The cells, concentrated in the pellet, were stored at -80°C until DNA extraction.

DNA extraction, library preparation and shotgun metagenomic sequencing

The DNA was extracted from the pellet using a bead-beating method, followed by the PowerFood® Microbial DNA Isolation Kit (MO BIO-Qiagen, USA). DNA was quantified using the BioSpectrometer® (Eppendorf, Milan, Italy) and then fragmented and tagmented with sequencing indexes and adapters using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). The Illumina sequencing platform NextSeq500 was used to perform the shotgun metagenomic sequencing in paired-end mode at 2×150 bp.

All shotgun metagenomic sequences tested in this study were deposited as FASTQ format in the Sequence Read Archive at the National Center for Biotechnology Information portal and are publicly available under the project named PRJNA1051357

Bioinformatic analysis

Raw data were processed with AdapterRemoval v2 (Schubert *et al*., 2016) to trim sequencing adaptors and poor-quality nucleotides at the reads tail. Cleaned sequences were aligned with Bowtie2 (Langmead *et al*., 2019) against the corresponding host genomes, either pig (susScr11), sheep (oviAri4) or chicken (galGal6) accessed at the UCSC Genome Browser (http://hgdownload.soe.ucsc.edu/downloads.html). Taxonomic profiling was performed with the web tool MG-RAST (Keegan *et al*., 2016). A suite of scripts from the MG-RAST-Tools repository was adopted to retrieve matrices of taxonomic abundance at phylum, class, order, family, genus and species levels. Shannon α diversity and Bray-Curtis β diversity indices were computed respectively with the R functions *diversity* and *vegdist* (package *vegan*). The correlation between taxa abundances was evaluated with the R package *corrplot*. After performing *de novo* assembly with SOAPdenovo2 (Luo *et al*., 2012), the scaffolds obtained were analyzed with a resistance gene identifier to identify and annotate ARGs (Alcock *et al*., 2023). All the heatmaps were obtained using the R package *ComplexHatmap* (clustering method: ward.D; clustering distance: canberra).

The estimation of reproducibility was carried out on each pair of technical replicates and the taxonomic profile overlapping rate (OvR) was calculated as in Equation 1:

$$OvR = \frac{Nc}{Nt} \quad \text{[Eq. 1]}$$

where Nc is the number of taxa concordant between the pair of samples, and Nt is the number of taxa with an average relative abundance ≥0.1%. The concordance criterion was defined as in Equation 2:

$$\frac{Abs\,(Ai-Aj)}{Ai+Aj} \cdot 2 < 0.25 \quad \text{[Eq. 2]}$$

Results

The sequencing experiment produced an average of 34.82×10^6 reads per sample ranging from a minimum (min) of 21.54×10^6 to a maximum (max) of 51.43×10^6, corresponding to a total of 67.9 giga bases of sequenced nucleotides slightly reduced after cleaning and trimming of the adaptors. The non-host sequences, retained after the host removal procedure, were highly variable with an average of 33%, a minimum of 0.14% for Merguez sausage, and a maximum of 97.98% for the Italian meat mixture IT4.

The taxonomic profiles, reported as relative abundances, highlighted a strong overlapping between technical replicates and high variability between different meats at both phylum and genus level (Figure 1A and B). The majority of bacteria detected in all the samples belonged to the phyla Firmicutes (mean=43.9%, min=6.13%, max=93.03%), Proteobacteria, mainly in Portuguese, Moroccan and EL1 Greek sausages (mean=16.92%, min=1.17%, max=92.52%), and Actinobacteria, mostly in Italian salami and EL2 Greek sample (mean=13.44%, min=0.00%, max=35.73%). A high abundance of the fungus Ascomycota (mean=18.79%,

min=0.01%, max=51.94%) was also detected in the 18-week cured Italian salami.

A greater complexity emerged when analyzing the taxa composition of the genera. *Lactobacillus* was the most represented genus (mean=14.81%, min=0.00%, max=64.37%), followed by *Staphylococcus* (mean=10.05%, min=0.03%, max=31.61%) that was found mainly in the Italian meats. The genera *Brevibacterium* (mean=5.71%, min=0.00%, max=16.53%), *Aspergillus* (mean=4.00%, min=0.00%, max=12.46%) and *Neosartorya* (mean=4.27%, min=0.00%, max=14.10%) were particularly represented in the Italian salami replicates, while *Macrococcus* (mean=4.49%, min=0.00%, max=41.45%) was peculiar in the 2 samples of Italian meat mixture. On the other hand, a higher abundance of *Enterococcus* (mean=3.52%, min=0.31%, max=18.03%), *Citrobacter* (mean=2.34%, min=0.00%, max=15.16%), and *Lactococcus* (mean=2.15%, min=0.00%, max=9.69%) was retrieved in Moroccan, Portuguese and Greek sausages.

The analysis of the α diversity showed that the genera richness (computed as Shannon index) ranged uniformly between 3.1 and 3.6 in all the samples except for IT3, IT5, and IT6 meats in which the value of α diversity was lower (2.0, 2.0 and 2.2 respectively). Focusing on the β diversity any separation emerged neither according to the meat origin nor to the curing time. However, the analysis showed that the diversity between the replicates is very small (data not shown). This observation agreed with the estimation of reproducibility performed on the taxonomic relative abundances. We estimated that all the replicates' pairs strongly correlated in terms of taxa abundances at both phylum and genus levels with a Pearson correlation coefficient (R^2) >0.98 in all the performed comparisons at both phylum (*i.e.*, IT1a-IT1b R^2=0.9985, IT1a-IT1c R^2=0.9941, IT1b-IT1c R^2=0.9948, IT2a-IT2b R^2=0.9990) and genus levels (*i.e.*, IT1a-IT1b R^2=0.9980, IT1a-IT1c R^2=0.9933, IT1b-IT1c R^2=0.9890, IT2a-IT2b R^2=0.9968). In addition, a large concordance was also found for the sample pairs at the genus level: 89.77% (79/88), 84.09% (74/88) and 80.68% (71/88) of concordance degree was computed for the couple of technical replicates IT1a-IT1b, IT1a-IT1c, and IT1b-IT1c respectively; while the couple IT2a-IT2b showed overlapping of 70 genera over 72 (97.22%).

Focusing on foodborne pathogens reported in Table 1, our metagenomic dataset showed that reads of *Escherichia*,

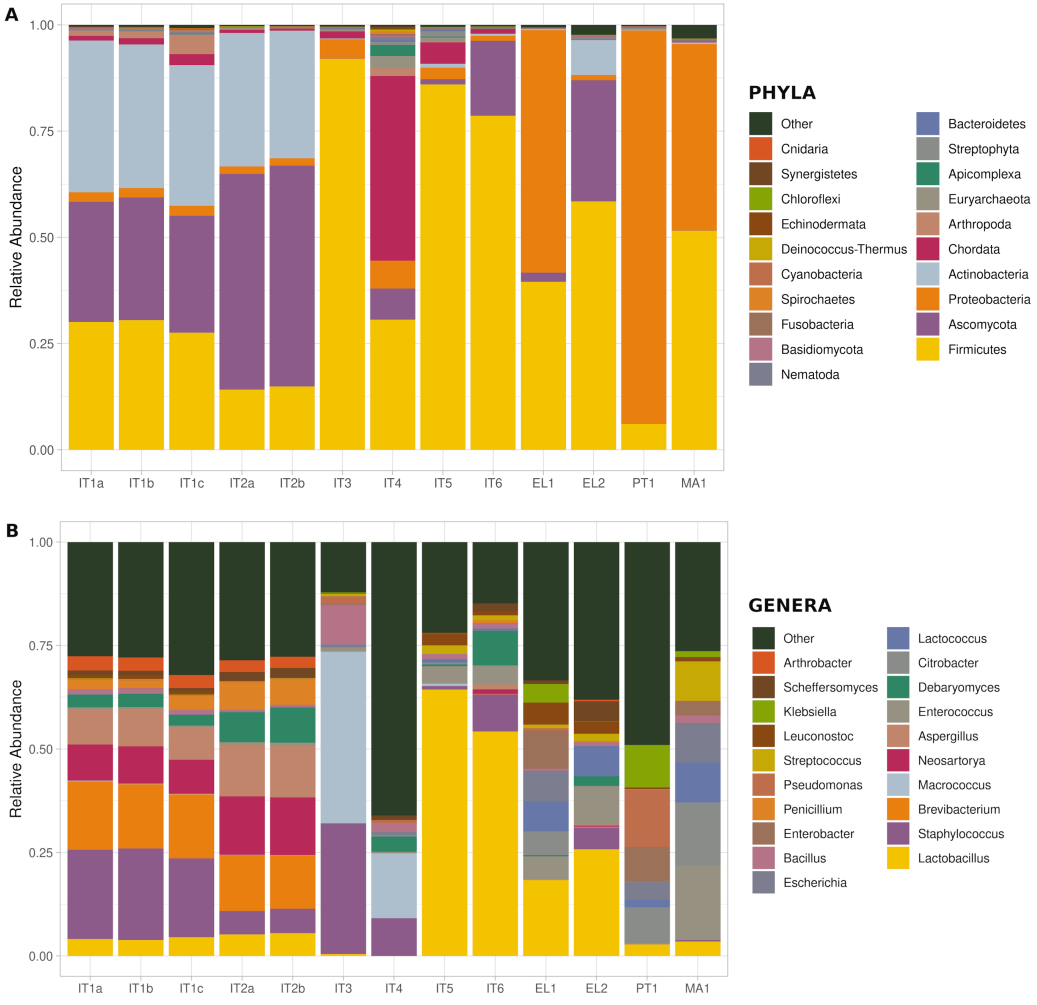

Figure 1. Taxonomic composition at phylum (**A**) and genus (**B**) levels. For both phyla and genera the 20 most represented taxa were plotted. IT, Italian meat; EL, Greek meat; PT, Portuguese meat; MA, Moroccan meat.

Salmonella, *Klebsiella,* and *Yersinia* were mainly found in meats from Greece, Morocco and Portugal, while *Staphylococcus* was present in Italian and Greek samples that also showed *Enterococcus*, *Bacillus* and *Streptococcus* together with Moroccan sausage with relative abundance >5% in at least one sample (Figure 2A). Among the detected species, several foodborne pathogens were identified: *S. aureus* (mean=1.52%) was found mainly in Italian meats, with a maximum relative abundance of 7.07% on meat mixture IT3 also showing the highest abundance of *B. cereus* (mean=0.18%, max = 0.8%); *E. coli* (mean=1.37%, max=7.29%) was detected in MA1; *S. enterica* (mean=1.22%, max=5.71%) was detected in EL1; *K. pneumoniae* (mean=0.99%, max=7.72% in PT1) and *Y. enterocolitica* (mean=0.25%, max=2.16% in PT1) were detected in PT1. The correlation plot in

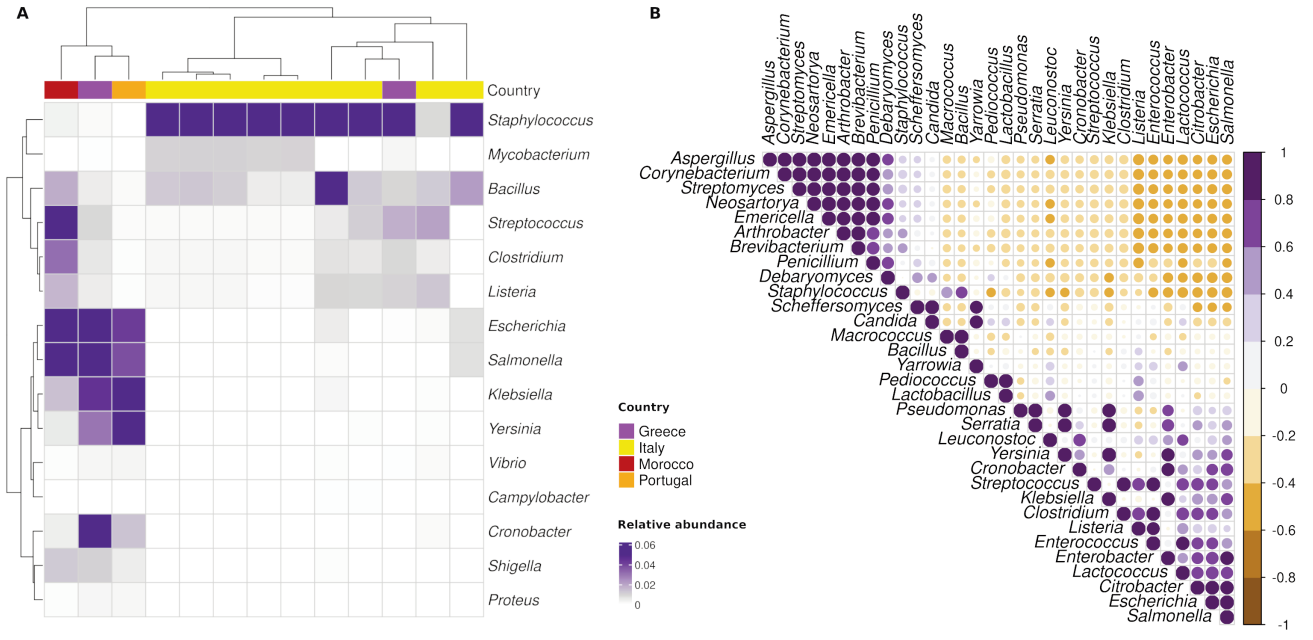

Figure 2. A) Heatmap and hierarchal clustering depicting the relative abundance of genera including also foodborne pathogens; the bottom barplot indicates the abundance of predicted virulence factors; **B**) correlation plot representing the correlation coefficients among genera. Positive and negative associations are highlighted in purple and orange respectively; the larger dot size corresponds to a greater statistical significance.

Table 1. Relative abundance (expressed as percentage) of the bacterial genera associated with foodborne diseases.

GENUS	IT1a	IT1b	IT1c	IT2a	IT2b	IT3	IT4	IT5	IT6	EL1	EL2	PT1	MA1
Staphylococcus	21.61	22.12	19.09	5.62	5.91	31.61	9.12	0.84	8.96	0.16	5.25	0.03	0.35
Klebsiella	0.02	0.01	0.01	0.01	0.01	0.23	0.00	0.07	0.02	4.50	0.02	10.22	1.41
Bacillus	1.22	1.31	1.13	0.47	0.49	9.60	2.19	1.19	1.20	0.49	0.90	0.13	1.85
Escherichia	0.02	0.02	0.02	0.01	0.01	0.48	0.73	0.14	0.03	7.47	0.05	4.22	9.51
Streptococcus	0.16	0.16	0.14	0.12	0.14	0.54	0.00	2.09	1.13	0.87	1.80	0.12	9.47
Yersinia	0.02	0.02	0.02	0.01	0.01	0.03	0.00	0.07	0.08	2.99	0.03	7.98	0.55
Salmonella	0.02	0.02	0.01	0.01	0.01	0.13	0.73	0.00	0.02	5.53	0.02	3.80	4.99
Clostridium	0.21	0.21	0.20	0.13	0.15	0.69	0.00	0.49	0.60	0.60	0.89	0.08	3.15
Listeria	0.21	0.20	0.20	0.10	0.09	0.90	0.00	1.32	0.89	0.48	1.03	0.07	1.63
Shigella	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	1.03	0.01	0.45	1.20
Mycobacterium	1.03	1.04	1.04	1.00	0.98	0.00	0.00	0.00	0.02	0.04	0.24	0.03	0.01
Vibrio	0.02	0.02	0.02	0.02	0.02	0.06	0.00	0.14	0.01	0.24	0.04	0.26	0.08
Campylobacter	0.02	0.01	0.02	0.01	0.01	0.04	0.00	0.00	0.04	0.01	0.02	0.01	0.02
Cronobacter	0.00	0.01	0.01	0.00	0.00	0.03	0.00	0.14	0.01	5.54	0.00	1.33	0.42
Proteus	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.03	0.23	0.01	0.20	0.07

IT1a, IT1b, IT1c (three replicates), IT2a, IT2b (two replicates), IT3, IT4, IT5 and IT6, Italian meats; EL1 and EL2, Greek meats; PT1, Portuguese meat; MA1, Moroccan meat

Figure 2B shows that the abundance of relevant foodborne pathogens, such as *Salmonella*, *Escherichia*, and *Listeria*, negatively correlated with the fungi *Aspergillus*, *Neosartoria*, *Emericella*, *Penicillum,* and *Debaryomyces*. We also found that the same fungi had an inverse association with some lactic acid bacteria (LAB) such as *Lactococcus*, *Enterococcus*, *Streptococcus*, *Leuconostoc*, and *Lactobacillus*.

The resistome analysis indicated that genes conferring resistance to aminoglycoside, macrolide, and tetracycline were widely spread in all the samples. Resistance to many other drug classes was also observed in specific samples: genetic determinants providing resistance against fluoroquinolone, penam, and cephalosporin agents were mainly found in the sample cluster containing MA1, EL1, and PT1; genetic determinants providing resistance to sulphonamide, acridine dye and to the class including disinfecting agents and intercalating dyes were identified in the Italian meats IT3-4-5-6. Finally, other relevant antimicrobial resistance determinants were identified but not linked to specific clusters of samples (Figure 3).

Discussion

In the present study, shotgun metagenomic sequencing was applied to determine the microbial composition, including the presence of foodborne pathogens, and the presence of ARGs in fermented meats produced in several Mediterranean countries.

The microbial profile of our sample's cohort showed appreciable variability in terms of both the quantitative and qualitative composition of microorganisms. The heterogeneity found in different types of fermented meats can be attributed to several factors, including the specific fermentation process, the type of meat used, regional variations in traditional practices, and the environmental conditions during fermentation that could influence the complex microbial-driven process that involves the growth and activity of bacteria, yeasts, and molds.

The dominant bacterial phyla found in our samples were Firmicutes, Proteobacteria, and Actinobacteria. This result and the detection of a fungal component, mainly constituted by

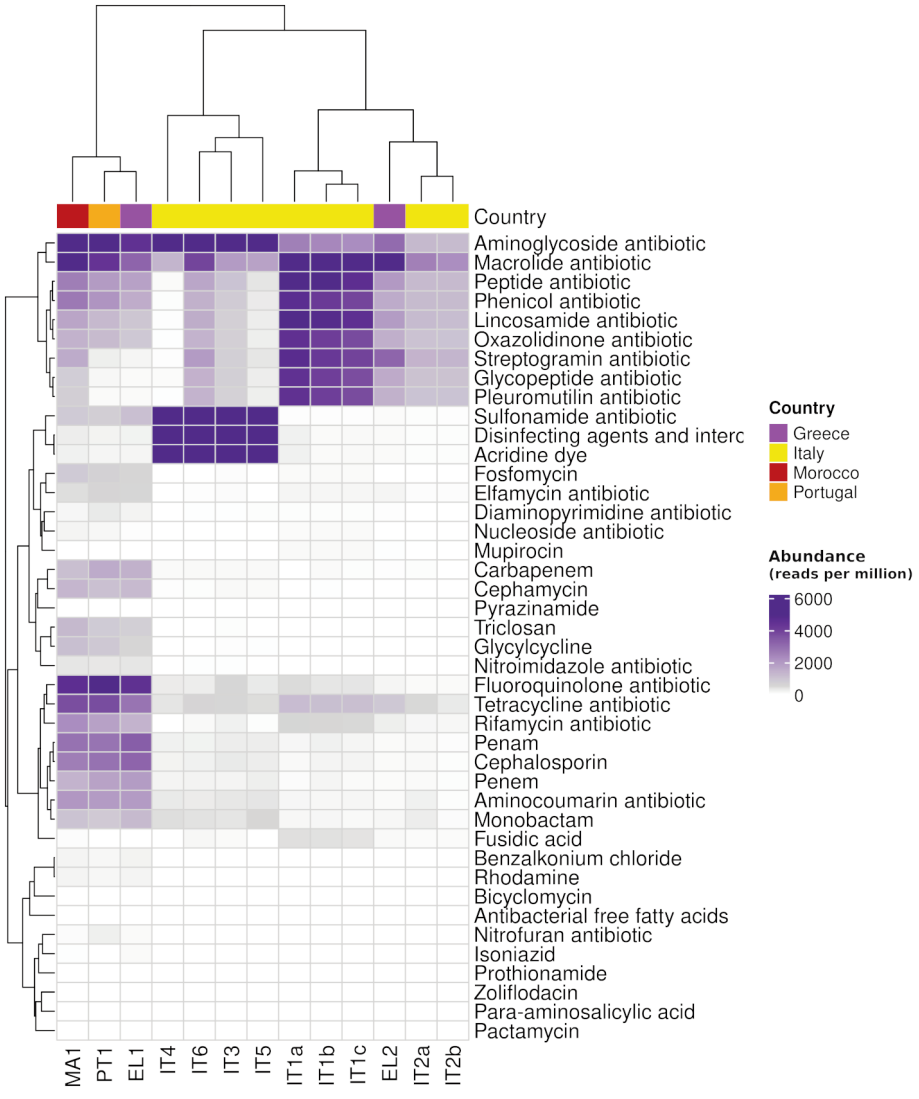

Figure 3. Abundance of sequencing reads mapping on antimicrobial resistance genes conferring resistance to the reported antibiotics and disinfecting agents. IT, Italian meat; EL, Greek meat; PT, Portuguese meat; MA, Moroccan meat.

Ascomycota, is in agreement with the literature (Wang *et al.*, 2018; Li *et al*., 2019; Wang *et al.*, 2021). Firmicutes is the phylum including LAB. Many LAB strains are well known for their role in meat fermentation, contributing to the formation of the typical quality features, like flavor, texture, and color, of salami and sausages (Połka *et al*., 2015; Cruxen *et al*., 2019;). Our data showed the presence of several LAB among the most represented genera of bacteria, including *Lactobacillus*, *Enterococcus* and *Lactococcus.* Their abundance was relevant in all samples, except for the 2 Italian meat mixtures which did not undergo any fermentation process. In the meat mixtures, we found Gram-positive cocci also common in fermented meat, namely *Macrococcus* and *Staphylococcus* (Franciosa *et al*., 2018; Zhang *et al.*, 2021).

Among the *Staphylococcus* spp. our samples showed the presence of *S. aureus* as well as non-pathogenic species (*i.e.*, *S. saprofiticus*, *S. epidermidis*). *S. aureus* is able to grow under a wide range of environmental conditions given its tolerance to salt and nitrite and has been implicated in staphylococcal food poisoning outbreaks associated with the consumption of fermented sausages (González-Fandos *et al*., 1999). In addition to *S. aureus*, reads of *E. coli*, *S. enterica*, *Y. enterocolitica*, *B. cereus*, and *K. pneumoniae* were also retrieved. Both *E. coli* and *S. enterica* were found with a relative abundance lower than 1% in the Italian salami but with a higher abundance in Greek, Moroccan, and Portuguese sausages. Shiga toxin-producing *E. coli* (STEC) and several serovars of *S. enterica* (such as Typhimurium, Newport, Kedougou, Goldcoast, and Bovimorbificans) were identified as etiologic agents in different meat-related outbreaks in which the foods responsible for illnesses were raw or cured fermented sausages (Omer *et al*., 2018). Unfortunately, we were not able to determine the pathogenicity of our *E. coli* by shotgun metagenomic since antigen genes cannot uniquely identify STEC serotype in a mixed sample. Greek, Moroccan, and Portuguese meats showed the presence of *K. pneumoniae* and *Y. enterocolitica* reads. These two organisms, both Gram-negative bacteria belonging to the Enterobacterales order, are a common cause of human infections. The primary mode of transmission of *Y. enterocolitica* is through the consumption of contaminated food or water; in particular, the main natural reservoirs are pig tonsils and feces that may contaminate the carcasses during slaughtering with subsequent introduction of the pathogen into the food chain (Terentjeva *et al*., 2022). On the contrary, the role of *K. pneumoniae* as a foodborne pathogen is still uncertain. A low abundance of *B. cereus* (<1%) was found in all the meat samples (except the IT4 meat mixture). *B. cereus* is a spore-forming bacteria able to cause foodborne gastrointestinal illnesses, but it was never associated with the consumption of fermented meat (Omer *et al*., 2018).

The fungi identified in our samples belonged to the genera *Aspergillus*, *Neosartoria*, *Emericella*, *Penicillum*, and *Debaryomyces*. During the ripening time, the dry-fermented sausages are naturally colonized by yeasts and molds on the external surface. Although some of them can cause human diseases (such as allergic reactions and fungemia), fungal infections associated with the consumption of food are uncommon (Delgado *et al*., 2023). On the other hand, several positive effects of yeasts and molds in meat fermentation are known, including the development of characteristic flavors and aromas in the final product, the contribution to the desired texture, and the role in the formation of a mycelial layer protecting the product from light and oxygen and preventing oxidation and rancidity processes and the growth of undesired microorganisms (Álvarez *et al*., 2023). Indeed, our correlation analysis showed that the fungal abundance was higher where the presence of foodborne pathogens and LAB was lower, indicating a negative correlation between the two microbial components. To explain this observation further studies will be planned.

Besides the taxonomic profiling, our shotgun metagenomic results enabled us to quantify the relative abundance of specific functional genes. The taxonomic biodiversity of fermented meats, from both pathogenic and non-pathogenic bacteria, may introduce numerous genetic elements, including ARGs (Zinno *et al*., 2023). Our dataset showed several AMR determinants conferring resistance to aminoglycoside, macrolide, and tetracycline in most samples. In addition, other specific resistance determinants were found in clusters of samples that were neither clearly associated with the country of origin nor with the other metadata available.

Besides taxonomic and functional gene characterization, in this study, we checked the reproducibility of shotgun metagenomic results when fermented food matrices are investigated. The evaluation was performed by computing the overlapping and the correlation between the taxa's relative abundances in two sets of technical replicates. The results showed that the reproducibility between technical replicates was very high under the same analysis conditions (either DNA extraction, library preparation, sequencing analysis, and bioinformatic analysis) considering both the degree of overlapping and the pairwise correlation. This finding may help the researchers in the phase of experimental design as it clearly suggests that it is not necessary to analyze technical replicates of the same sample and that sequencing of a single biological sample is sufficient.

Conclusions

This pilot study compares the taxonomic composition and presence of AMR genes in artisanal fermented meat products collected in different Mediterranean countries. Moreover, it provides a first insight into the reproducibility of metagenomic results obtained by sequencing technical replicates of the same biological sample.

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