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Comparative analysis of the microbiome composition of artisanal cheeses produced in the Mediterranean area

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Abstract
In the PRIMA project ArtiSaneFood, the microbiological parameters of several artisanal cheeses produced in the Mediterranean area have been quantified. In this pilot study, we selected four of these artisanal cheese products from Italy, Portugal, Spain, and Morocco to investigate and compare their microbiomes in terms of taxonomy composition, presence of reads of foodborne pathogens, as well as virulence and antimicrobial resistance genes. *Lactococcus*, *Streptococcus* and *Lactobacillus* were the most represented genera in the Portuguese and Spanish cheeses, *Streptococcus* in the Italian cheese, and *Enterococcus*, *Klebsiella*, *Escherichia,* and *Citrobacter* in the Moroccan products.
The correlation analysis indicated a negative association between the abundance of some lactic acid bacteria (*i.e.*, *Lactococcus*, *Lactobacillus*, *Streptococcus,* and *Leuconostoc*) and foodborne pathogenic genera, like *Escherichia* and *Salmonella.* The analysis of pathogen abundance, virulence factors, and antimicrobial resistance genes showed a strong clusterization based on the cheese type, confirming that the presence of potential human health risk determinants was higher in the artisanal products derived from unpasteurized milk that underwent spontaneous fermentation.

Introduction
Artisan cheeses are highly appreciated for their genuineness and organoleptic properties. Unlike industrial dairy products, artisanal cheeses are made following traditional practices implemented according to the cheese producer's experience. As a consequence, during the production process, the time or temperature of single steps might slightly change based on the judgment of the producers assessing the aspect of the products. The use of manual procedures and the lack of strict process parameter control all over the process can make artisanal cheeses more prone to contamination, survival, and growth of foodborne pathogens in comparison to industrial products (Rangel-Ortega *et al.*, 2023). Some artisanal cheese producers in the Mediterranean area prefer to use raw milk instead of pasteurized milk. The pathogens that can be detected in raw milk and which are normally inactivated by pasteurization, have been summarized by EFSA (2015) and are mostly represented by *Escherichia coli*, *Salmonella*, *Listeria monocytogenes,* and *Staphylococcus* aure*us.* These pathogens can survive in the final cheese product if not properly handled (Yoon *et al*., 2016).
In 2022, 30 strong evidence outbreaks, corresponding to 6.2% of the total, were vehiculated by milk and milk products in European countries (EFSA, 2023) but there are no data on artisanal cheeses as possible sources. A less recent US study reported that during 1998-2011, 38 outbreaks were due to artisanal cheeses made with unpasteurized milk (42% of the total), remarking the risk of consuming unpasteurized dairy products, especially for vulnerable consumers, like older adults, pregnant women, and immunocompromised individuals (Gould e*t al*., 2014).
The microbiological safety of artisanal cheeses is certainly affected by the microbiological composition of the raw milk, the level of hygiene in the equipment and environment of the production facility, the type of process, and the staff skills. However, the occurrence, survival, and growth of foodborne pathogens in artisanal cheeses are also affected by the interplay between pathogens and the indigenous microbiota, such as lactic acid bacteria (LAB). LAB usually represent the most abundant bacterial population in artisanal cheeses and are able to produce a variety of antimicrobial compounds against foodborne pathogens (Campagnollo *et al*., 2022).
The dynamics between LAB, or other beneficial microbial populations, and foodborne pathogens in cheese can be investigated by shotgun metagenomics. Compared to culture-based approaches, which only capture a small portion of the cultivable microbial community, this sequencing technology offers the opportunity to identify all the microbial populations in the cheese, including those represented by non-cultivable or difficult-to-culture microorganisms. Besides the taxonomic profiling, shotgun metagenomic analysis offers also insights into the microbial community's functional genes, which is essential to understanding functions and interactions between microorganisms within a food ecosystem (Handelsman, 2004). Despite these advantages, metagenomic is also characterized by some weaknesses, limiting its implementation in food and

foodborne outbreak investigations, such as a lack of standardized methodologies for the wet lab part and of open access bioinformatic pipelines for the sequence analysis (Koutsoumanis *et al.*, 2019).
In the PRIMA project ArtiSaneFood, the microbiological parameters of a number of artisanal cheeses have been quantified (Faria *et al.*, 2022; Pasquali *et al.*, 2022; Possas *et al.*, 2022). In this pilot study, we selected four of these artisanal cheeses to further investigate and compare their microbiomes in terms of taxonomy composition, presence of reads of foodborne pathogens as well as virulence and antimicrobial resistance genes.

Materials and Methods

Investigated samples and shotgun metagenomic sequencing

In this pilot study, 12 cheese samples were analyzed by shotgun metagenomic. The samples were represented by *Squacquerone* produced in Italy (n=1), *Jben* produced in Marocco (n=3), *Queso Manchego* produced in Spain (n=1) and *Queijo de Cabra Transmontano* produced in Portugal (n=7).
The *Squacquerone* was the only cheese produced with filtered and pasteurized milk, while raw milk was employed in all the other three artisanal cheese preparations. In *Trasmontano*, *Manchego*, and *Squacquerone* cheeses, the fermentation process and coagulation were supported by the supplementation of starter cultures and rennet, while Jben underwent spontaneous fermentation.
For the metagenomic analysis, 10 g of cheese were diluted in 40 mL of sterile physiological solution (0.90% NaCl) and homogenized using a stomacher (MAYO HG 400V, Stomacher, Baranzate, Italy) at a normal speed for 1 minute. All the solutions of the homogenized samples were centrifuged at 9980× g for 20 minutes at 4°C to collect the cells inside the pellet, which was kept at -80°C until DNA extraction.
To extract DNA from the stored pellets, the PowerFood® Microbial DNA Isolation Kit (MO BIO-Qiagen, Hilden, Germany) was used. The extracted DNAs were quantified with the BioSpectrometer® (Eppendorf, Milan, Italy) and then fragmentation, tagmentation and indexing were performed using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Shotgun metagenomic sequencing was carried out using the Illumina sequencing platform NextSeq500 in paired-end mode at 2×150 bp.

Bioinformatic and statistical analysis

Bioinformatic analysis was carried out as previously described (Indio *et al*., 2024). Briefly, the raw sequences were trimmed with AdapterRemoval v2 to remove sequencing adaptors and low-quality tails. Following cleaning, sequences were aligned using Bowtie2 (Langmead *et al.*, 2019) to the appropriate host genomes, extracted from the UCSC Genome Browser resource (http://hgdownload.soe.ucsc.edu/downloads.html) for cow (bosTau9), sheep (oviAri4), and from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/datasets/genome/) for goat (ARS1.2).
The web tool “Metagenomics Rapid Annotation using Subsystem Technology” (MG-RAST) was adopted to perform the taxonomic profiling (Keegan *et al.*, 2016), the quantification at the phylum, class, order, family, genus and species levels was retrieved using a suite of scripts included in the MG-RAST-Tools repository.
The R package vegan was used to compute the Inverse Simpson (Fisher) α diversity and Bray-Curtis β diversity indices (respectively with the functions of *diversity* and *vegdist*), while *corrplot* was adopted to evaluate the correlation between taxa relative abundances. Principal coordinate analysis was performed with *cmdscale* (*stats* package) and plotted as 3D projections with *rgl* package (function *plot3d*). The raw sequences were also *de novo* assembled with the algorithm SOAPdenovo2 (Luo *et al*., 2012) using the command *SOAPdenovo-63mer* and setting the options "*-K 55 -p 16 -M 3 -F -u*". The obtained scaffolds were analyzed with Resistance Gene Identifier to identify and annotate ARGs (Alcock *et al*., 2023), and the presence of virulence factors were checked using *blastn* against the Virulence Factors Database (Liu *et al*., 2022) and quantified as

transcript per million with the program Kallisto (Bray *et al*., 2016). All the heatmaps and the corresponding hierarchical clustering were obtained using the R package *ComplexHeatmap*.
The Wilcoxon rank-sum test for comparison of the mean values was applied using the Mann-Whitney U test calculator available at the Statistics Kingdom website (http://www.statskingdom.com/170median_mann_whitney.html). The statistical analysis was carried out to compare groups containing a number of samples equal to or greater than 3.

Results

The shotgun metagenomic sequencing runs on the tested samples produced 60.5 Gbases, corresponding to an average of 33.6 million reads per sample after trimming and adapters removal. Raw sequences obtained by shotgun metagenomic are publicly available as fastq format at the Sequence Read Archive, available on the NCBI server, under the project named PRJNA1117640. The presence of the host genome (either of goat, sheep, or cow) was very heterogeneous among the cheese samples, ranging between 67% in the *Squacquerone* to less than 1% in the *Jben* from Marocco (*Supplementary Table 1*).
The taxonomic evaluation showed clear differences in the composition of the microbial communities among the cheese types for both phylum and genus level classification. Firmicutes, followed by Proteobacteria and Ascomycota, was the most abundant phylum in all the cheeses, except for *Jben* in which Proteobacteria was significantly higher (p=0.012) and Ascomycota was <<1% (Figure 1A). At the genus level, *Lactococcus*, *Streptococcus* and *Lactobacillus* were the most represented genera in the Portuguese and Spanish cheeses, while the Italian one was characterized by the presence of *Streptococcus* and the Moroccan showed a high abundance of *Enterococcus*, *Klebsiella*, *Escherichia* and *Citrobacter*. Average abundances >2% of *Enterobacter*, *Pseudomonas*, and *Acinetobacter* were also highlighted in the dataset, even if not associated with specific cheese types (Figure 1B). Overall, the genera composition was uniform within the same type of cheese, as highlighted by the analysis of β diversity (Figure 1C), with a greater richness in the Moroccan cheese also highlighted by α diversity index calculation (p=0.009) (Figure 1D).
Our data showed the presence of reads of potentially pathogenic species in all the analyzed cheese samples. Interestingly, the unsupervised cluster analysis showed very distinct profiles related to the cheese type (Figure 2A). In particular, the highest relative abundance of pathogenic genera (such as *Escherichia*, *Salmonella*, *Klebsiella* and *Shigella)* was highlighted in the *Jben* cheese even if not statistically significant (p=0.064), while *Squacquerone* almost exclusively hosted *Streptococcus* genus, mainly represented by the non-pathogenic *S. termophilus*. *Enterococcus* was present in both Moroccan and Iberic cheeses, while the relative abundance of *Yersinia*, *Clostridium* and *Bacillus* were <1% in all the tested samples. Among the species belonging to these genera, our data showed several foodborne pathogens with an average abundance >1%, including *Salmonella enterica*, *E. coli*, *Klebsiella pneumoniae* (*Supplementary Table 2*).
The correlation analysis showed a negative association between the abundance of some LAB (*i.e*., *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Leuconostoc*) and the reads of foodborne pathogenic genera, like *Escherichia*, *Salmonella,* and *Klebsiella* (Figure 2B). The search for virulence factors highlighted distinct profiles related to cheese origin. In agreement with the presence of reads for zoonotic agents, the Jben showed the highest abundance of virulence factors (p=0.018), while *Squacquerone* showed the lowest. The most represented virulence factor category, found in all the samples, was “adherence” followed by “immune modulation” and “exoenzyme” which were more abundant in Iberic and Italian cheeses with respect to Moroccan that, conversely, were richer in virulence factors related to motility, biofilm, regulation, antimicrobial activity, nutritional and metabolic processes (Figure 3). Among the most abundant virulence factor genes in *Jben*, we found *fur*, *rpoS* and *nmpC* related to *S. enterica*, and the family of fimbrial genes from *Citrobacter freundii, wcalJ* and *ompA* belonging to the genome of *K. pneumoniae* and *E. coli,* respectively. Many other virulence factors related to the genome of *Streptococcus spp*. (such as *plr/gapA*, *cshA*, *rfbB*, *tig*/*ropA, cbpD*, and *eno*) were retrieved in *Squacquerone*, *Manchego* and *Trasmontano*; the

latter, in addition, showed several virulence genes carried by *Enterococcus faecalis,* including *bee2*, *str1* and *str2* (data not shown).
The analysis of antimicrobial resistance genes suggested a strong cauterization of *Jben* sample (Figure 4A) where a higher abundance of antimicrobial resistance (AMR) genes was highlighted (p=0.009). Here we detected AMR genes that are known to confer resistance to several antimicrobial agents, including β-lactam antibiotics (*i.e.* cephalosporin, cephamycin, penam, penem, monobactam, carbapenem), sulfonamide, rifamicyn, fosfomycin, tetracyclin antibiotics. Conversely, resistance to lincosamide, eflamycin, fusic acid and glycopeptide antibiotics was evidenced in the Portuguese and Spanish cheeses. In addition, genes associated with the resistance to antimicrobial peptides, fluoroquinolone and aminoglycoside antibiotics were detected homogenously through all the samples. Interestingly, we found that the genes coffering antimicrobial resistance in *Jben* cheese mostly derived from *E. coli*, *K. pneumoniae*, *S. Enterica* and many other less represented bacterial species, while the other chesses showed AMR genes associated to *L. monocytogenes*, *Bacillus cereus*, *Lactococcus lactis*, *S. aureus* and *Enterococcus faecium* (Figure 4B).

Discussion

In this pilot study performed on a limited number of samples but sequenced at a high coverage (*Supplementary Table 1*), the shotgun metagenomic approach was adopted to evaluate the microbiome composition of four different artisanal cheeses produced in the Mediterranean area.
Beyond the geographical origin, the cheeses differed also for the manufacturer procedures. Such differences were reflected in the richness and diversity of the microbial populations. As a matter of fact, the Italian cheese obtained from filtrated and pasteurized milk showed lower α diversity with respect to the products made with raw milk. In this regard, Walsh *et al*. (2020) reported that pasteurization of the milk did not have a significant impact on the α diversity of cheeses. In our dataset, a lack of statistics (due to the presence of a single cheese sample derived from pasteurized milk) did not allow us to determine the significance of the difference, although it is clearly evident (Figure 1D). As far as the raw milk-derived cheeses are concerned, the highest microbial richness (supported by statistics) was found in the *Jben* which underwent spontaneous fermentation, unlike the Portuguese and Spanish cheeses in which the raw milk was inoculated with started cultures. This evidence was previously described in a metagenomic study in which several fermented foods were analyzed, and the authors reported that the α diversity of spontaneously fermented foods was significantly higher than that of foods produced using starter cultures (Leech *et al*., 2020).
Our metagenomic dataset showed that the four cheeses analyzed had very distinct microbial profiles based on their geographical origin. Moroccan cheese differed significantly from all the others due to the dominance of coliform bacteria belonging to *Klebsiella*, *Escherichia,* and *Citrobacter* genera.
The presence of these microorganisms had previously been described in Moroccan cheese *Jben* and associated with inadequate hygienic conditions of preparation of this product or the poor bacteriological quality of raw milk used for this preparation (Hamama and Bayi, 1991; Azzouz *et al*., 2024). Conversely, the other cheeses showed a microbial composition mostly represented by LAB. In particular, *Lactococcus* and *Lactobacillus* were the main LAB populations in the Portuguese and the Spanish cheeses, while a predominance of *Streptococcus* (mainly *S. thermophilus)* was found in the Italian product (over 69%).
This result was expected because the guidelines to produce *Squacquerone di Romagna* allow to use of started culture *S. thermophilus* only (Aquilanti *et al*., 2012). The presence of LAB was widely described in artisanal cheeses (Quintana *et al*., 2020; Walsh *et al*., 2020), as well as in industrial dairy products where specific strains of LAB were used as starter according to their functional and organoleptic properties, such as the milk acidifying capacity, aromatizing power and the capability to inhibit the undesirable microorganisms (Thierry *et al*., 2015). The predominance of LAB in our metagenomic analysis is fully in line with what was reported in microbiological studies conducted on *Trasmontano* (Faria *et al.*, 2022), *Manchego* (Cabezas *et al*., 2007) and *Squacquerone* cheese

(Siroli *et al*., 2020). In this regard, also our correlation analysis indicated an inverse relationship between LAB and pathogens. We found that the relative abundance of pathogenic bacteria genera, like *Salmonella* and *Escherichia*, negatively correlated with the LAB component (such as *Lactococcus*, *Lactobacillus,* and *Streptococcus*). This interaction could be due to competitive exclusion (where LAB outcompete pathogens for nutrients and space) and the pathogen growth inhibition mediated by the production of lactic acid and/or antimicrobial agents (bacteriocins, hydrogen peroxide, ethanol) (Adams and Nicolaides, 1997). In our dataset, a higher presence of reads associated with pathogens was found in *Jben* cheese where, as a matter of fact, the raw milk was subjected to fermentation without the addition of starter culture and the abundance of LAB was the lowest. In this Moroccan product, we found a relevant presence of reads of *S. enterica, K. pneumoniae*, and *E. coli*. While *S. enterica* and Shiga toxin-producing *E. coli* were widely reported among the main pathogenic bacteria isolated from raw milk and artisanal cheeses that are associated with food-poisoning outbreaks (Baylis, 2009; Rangel-Ortega *et al*., 2023), *K. pneumoniae* was lesser known to cause foodborne illness although it is considered a zoonotic pathogen which can originate from a variety of sources in the dairy farm environment including water, plant materials, equipment, dirt, and fecal sources (Martin *et al*., 2016). However, the presence of multi-drug resistant *K. pneumoniae* strains in food constitutes a potential risk to public health, not only as a pathogen but also indirectly as a vehicle of AMR genes that could be passed to other pathogens in the microbial community of food and the human gut (Hartantyo *et al*., 2020). Indeed, our analysis of resistome showed that a significant proportion of antimicrobial resistance determinants in Moroccan cheese were linked to the presence of genes belonging to the genome of *K. pneumoniae* together with *E. coli* that, in fact, is also well known to represent one of the most important reservoirs of AMR genes in raw milk cheeses (Ünlü *et al*., 2011; dos Santos *et al*., 2022;).

Besides the reads of the pathogens described above, detected in *Jben*, additional reads associated with the genera *Listeria*, *Yersinia*, *Enterococcus*, *Bacillus* and *Clostridium* were found in the other cheese samples, except for *Squacquerone* cheese which was the only artisanal product obtained from previously filtered and pasteurized milk. However, it is important to highlight that the relative abundance of foodborne pathogens such as *L. monocytogenes*, *Y. enterocolitica*, *B. cereus* and *C. botulinum* was always lower than <1%. On the contrary, *Enterococcus* spp. showed an average abundance of 7%, representing the fourth most prevalent microbial group detected in our experiment. *Enterococci* (*i.e*., *E. faecalis*, *E. faecium*) have recently gained relevance as leading opportunistic pathogens causing various infections, including urinary tract diseases, bacteremia, meningitis, wound infections, and biofilm-associated infections of artificial medical devices that could be also referred to the consumption of contaminated cheese (Anderson *et al*., 2016). The only zoonotic agent that was present in all the tested cheeses was *Streptococcus spp*. However, only a very small proportion of the identified *Streptococci* belonged to foodborne pathogenic species, such as *S. agalactiae* and *S. equi,* both quantified in our samples with a relative abundance <1%.

Alongside the study of microbial composition, we investigated the presence in the tested cheeses of genes known to act as virulence factors and AMR genes. Both these two gene categories were higher in the samples where the pathogens were also higher.

This is not surprising since in the host-pathogen interaction the expression of virulence genes allows the bacteria to grow, spread, and cause host damage. In addition, the persistence of such pathogens in the food matrix is strongly related to the ability to escape the action of antimicrobials (Pérez-Rodríguez and Mercanoglu Taban, 2019). In the Moroccan cheese, we observed a wide range of potential virulence factors and AMR genes, mostly in *K. pneumoniae*, *S. enterica* and *E. coli.* These virulence genes were linked to bacterial adherence, biofilm production, motility, and multi-drug resistance mechanism. Conversely, the Iberic cheeses showed a high abundance of virulence determinants from *E. faecalis* and *Streptococcus* spp., but most AMR genes were associated with other bacteria genera (such as *L. monocytogenes, B. cereus, L. lactis*). On the other hand, the Italian product showed very little presence of AMR and virulence determinants, mainly associated with the presence of the starter LAB *Streptococcus thermophilus*.

Conclusions

This study offers a preliminary overview of the taxonomic composition, presence of virulence factors, and AMR genes in artisanal cheese products collected in different Mediterranean countries. The microbiomes associated with the tested products clustered according to the cheese origin confirming the impact of the production process, raw materials, and production environment on food microbiomes. Reads of foodborne pathogens were identified in all the productions and were negatively correlated to the relative abundance of LAB. Fighting foodborne pathogen colonization and modulating their interaction with LAB and other beneficial microbes certainly represents one of the most promising strategies to improve the safety of artisanal productions in the future.

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Online supplementary material:
Supplementary Table 1. Samples table with product characteristics and sequencing yield.
Supplementary Table 2. Relative abundance of bacteria species belonging to pathogenic genera. Only species with relative abundance >0.1% in at least one sample were reported.

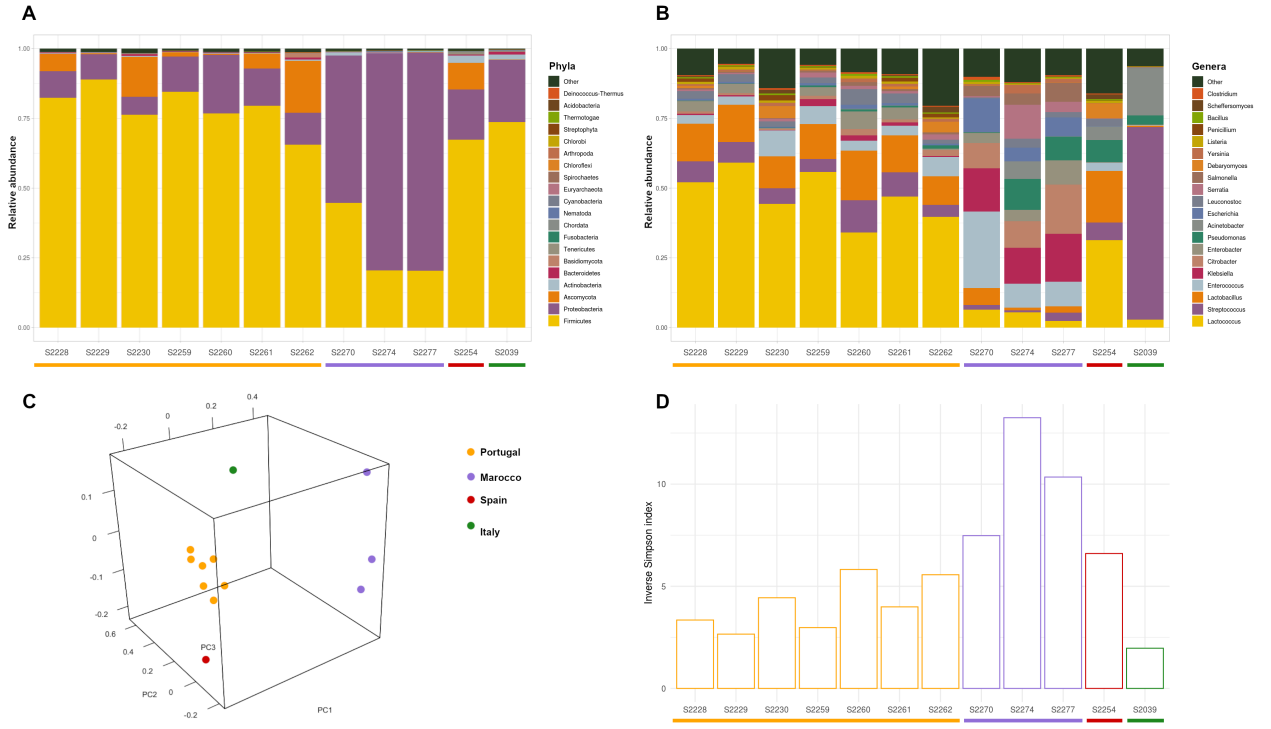

Figure 1. A) Relative abundance of the 20 most represented phyla; B) relative abundance of the 20 most represented genera; C) 3D projections of principal coordinate analysis obtained from Bray-Curtis β diversity calculation; D) inverse Simpson α diversity index.

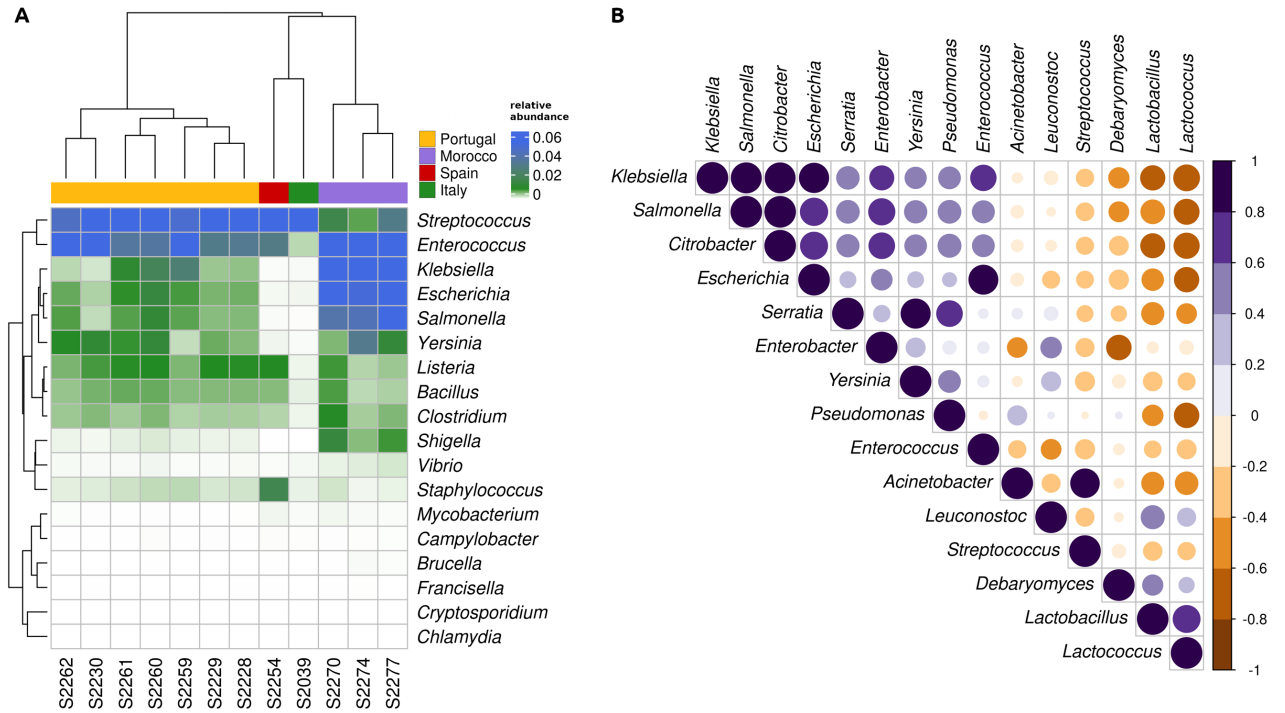

Figure 2. A) Heatmap representing the relative abundance of reads of pathogenic genera; rows and columns dendrograms derive from the hierarchical clustering analysis (method: ward.D2, distance: canberra); B) correlation plot representing the association between the relative abundance of the 20 most represented genera: purple and orange dots refer respectively to positive and negative correlation; the larger the dot, the greater the statistical significance of the association.

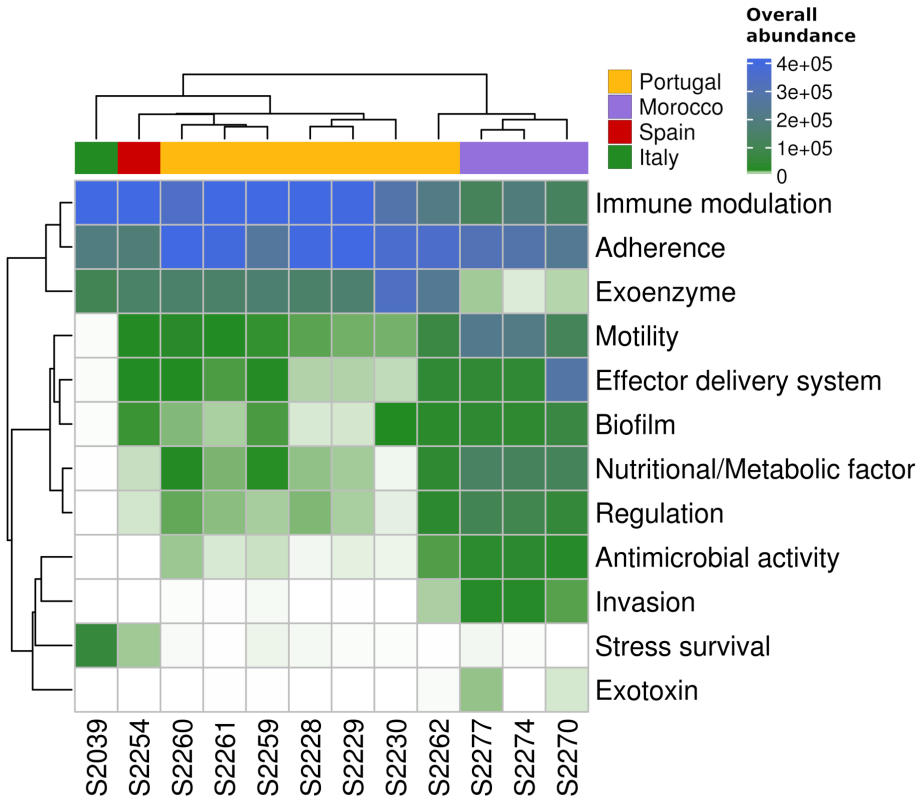

Figure 3. Heatmap representing the abundance of virulence factors; rows and columns dendrograms derive from the hierarchical clustering analysis (method: ward.D2, distance: canberra).

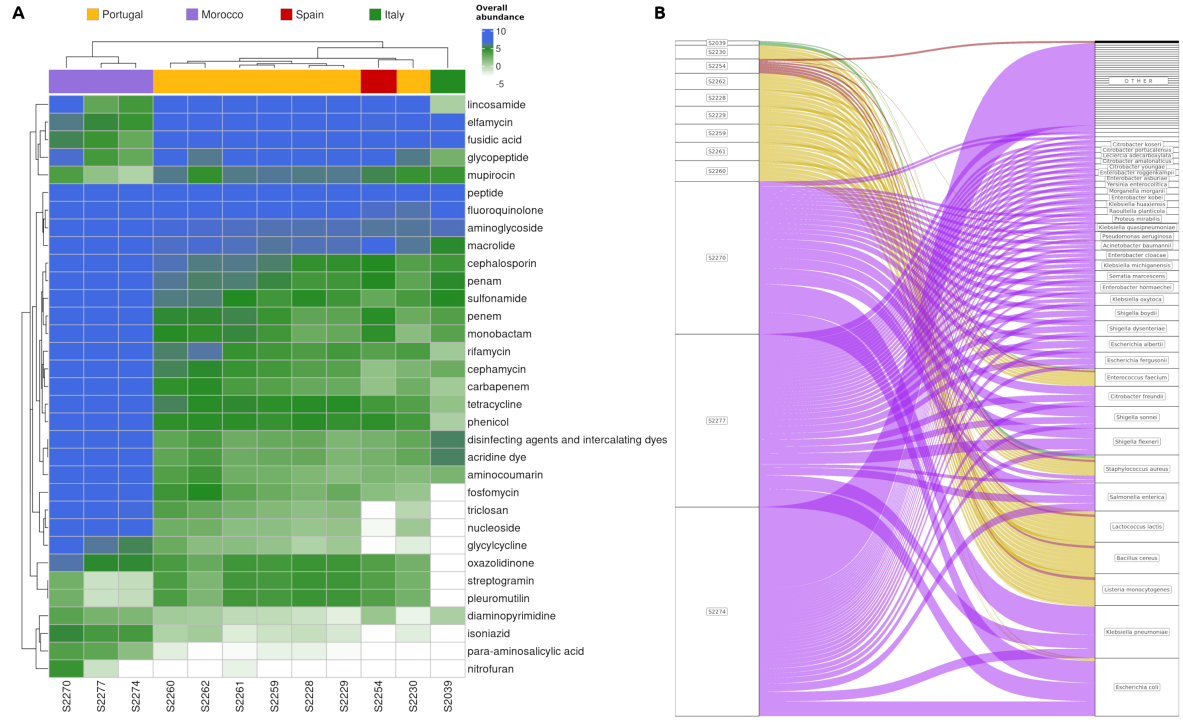

Figure 4. A) Heatmap representing the relative abundance of reads associated with genes conferring resistance to antimicrobial agents; rows and columns dendrograms derived from the hierarchical clustering analysis (method: average, distance: euclidean); B) alluvial diagram illustrates the bacteria species associated with the antimicrobial resistance in each sample.