UNIVERSITÀ CATTOLICA DEL SACRO CUORE

Sede di Piacenza

Ph.D. in Agro-Food System

Cycle XXXVI
S.S.D. AGR/16

Exploring Pathogenic Bacteria Dynamics and Antimicrobial Resistance Gene Transfer Across the Food Chain: Significance for Food Safety and Public Health.

Coordinator:
Ch.mo Prof. Paolo Ajmone Marsan

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Matriculation n: 5014538

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CHAPTER 1

General introduction: Achieving food safety and quality

An integrated approach for surveillance, detection and mitigation of pathogenic and antimicrobial resistant bacteria along the food chain.

1. The current microbiological safety and quality status of foods

Foods typically harbour a diverse array of microbial associations, largely influenced by the microbial load present in the raw materials[1]. Throughout the entire food process, from the initial handling to the manufacturing process and the final product, microbial diversity can be shaped and influenced[2]. This depends on several factors, such as the ability of the food to support microbial growth, the interactions between microorganisms, their interaction with the food, and the specific type and conditions of processing and storage environments[3–5].

Microbes associated with food can be categorized into two distinct groups based on their positive or negative impact on it[6]. Positive microorganisms are employed in specific production processes for their ability to provide desirable sensory and texture characteristics and extend the shelf life of the final food product. These pro-technological microorganisms are commonly applied in the manufacturing of fermented foods, including dairy products, meat products, alcoholic beverages, and bakery products[7]. Consequently, microorganisms deliberately added as starter cultures drive the fermentation of food, sometimes interacting with the microbiota of raw materials, inducing significant changes in the chemical, physical and microbiological composition of the food[8]. As a consequence, fermented food products acquire a distinct nutritional, sensory and health-promoting profile[9]. In particular, lactic acid bacteria (LAB) are considered to be the main contributors in food fermentation, thus promoting continuous efforts of isolating novel LAB strains with better performance in improving the characteristics and safety of fermented foods[10]. Extensive research has been conducted on many LAB strains, not only for their technological role in fermentation but also for their potential use as probiotics. Probiotic strains possess the ability to withstand the challenges of the gastrointestinal environment, adhere to the intestinal mucosa, and exhibit immunomodulatory activity[11,12]. In the European regulatory framework, novel LAB

species to be used as starters, probiotics in food must go through pre-market safety assessment process by European Food Safety Authority (EFSA). This also applies e.g. to all microbes used in the feed additives in the EU. To assist in the process QPS (Qualified Presumption of Safety) approach has been developed[13]. QPS approach assesses the safety of microbes based on their taxonomic definition, pathogenicity potential (including virulence and antibiotic resistance), human/animal exposure, and expected use. Being able to apply the QPS approach in the safety assessment streamlines the pre-market assessment process considerably. However, certain microbial genera like *Enterococcus*, despite some strains possessing favourable technological and probiotic properties, have not obtained QPS status due to issues related to potential pathogenicity and presence of acquired antimicrobial resistance genes. Any microbial species not mentioned in the QPS list must go through more rigorous safety assessment, described in various EFSA guidances. Since correct taxonomic identification using molecular tools is the basis of the safety assessment development of tools that enable rapid and reliable is necessary[14].

In additions to LABs, yeasts also perform a crucial role in food fermentation, especially in the production of beer, wine and bread. Many yeast strains have been isolated from naturally fermented foods and then studied and used as starters in fermentation processes. In addition to their ability to conduct fermentations, yeasts and moulds are widely used as producers of enzymes, which can be used as food and feed additives[15].

Saccharomyces cerevisiae var. *boulardii*, and certain species belonging to *Debaryomyces, Hanseniaspora, Pichia, Meyerozyma, Torulaspora* are potential probiotics[16]. However, some yeast species can negatively affect the quality of foods by spoiling it. Yeast strains deliberately added to food go through a similar safety assessment approach as described above for LAB[14].

Foods provide a suitable substrate for the growth of many harmful microorganisms, including spoilage-causing and some pathogenic microorganisms. Microbial proliferation during food spoilage results in the production of metabolites that affect the nutritional quality, the texture, and sensory properties of the food products. Spoilage is defined by the presence of volatile compounds that result in off-flavours and/or package swelling, along with visual deterioration of the foods[17,18]. Although processing technologies focused on improving shelf life and preserving food characteristics are constantly evolving, spoilage remains a global problem and the leading cause of food losses[19].

The presence of spoilage microorganisms, such as bacteria, yeasts, and moulds, primarily depends on the specific attributes of the food matrix and the processing environment. Food products characterized by high water content, such as meat and milk-based products, are primarily susceptible to bacterial growth[20]. In contrast, foods with a low free water content, such as bread, are more prone to yeast and mould contamination and proliferation[21]. The intricate nature of food spoilage and the numerous contributing factors make it a challenging problem to address. Consequently, to minimize the presence of spoilage microbes effectively a thorough analysis of the food matrix is crucial in determining the most appropriate processing technologyto be applied [22,23].

Although microbial spoilage causes product alteration resulting in unsuitability for consumption and food loss. Food legislation only regulates the presence of pathogenic microorganisms in food, which can cause foodborne illnesses in humans. Therefore, food safety focuses on the control and prevention of microbial hazards in food in order to preserve the health of consumers.

Furthermore, the variety of stakeholders involved in the food production and consumption result in an increasingly complex food chain that needs to accommodate different lifestyles and dietary habits. Additionally, globalization of the food systems necessitates integrated collaboration between all steps of the production chain to ensure adequate food quality and safety. Given that pathogen contamination can occur in multiple steps within the food chain, it is essential for each production step to adhere to the hygiene regulations and preventive measures established by the Hazard Analysis and Critical Control Point (HACCP) system, aiming to mitigate the risk to human health[24].

The integration and collaboration among the different animal, human and environmental sectors involved in food production, allows the problem of food safety to be addressed using a One Health approach[25]. Foodborne illnesses, arising from the ingestion or direct contact with contaminated food, are often linked to zoonotic agents, environmental pathogenic microorganisms that can be transmitted between animals and humans. Pathogenic micro-organisms such as *Salmonella enterica*, *Campylobacter coli/jejuni*, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Escherichia coli* are mainly transmitted through food ingestion while, *Brucella* spp., enterohaemorrhagic *Escherichia coli* (EHEC), Methicillin-resistant *Staphylococcus aureus* (MRSA) infect humans mainly through direct human to human contact[26].

Pathogenic microorganisms in the environment largely originate from animal excretions, making the environment a reservoir and a potential source of pathogens for both domesticated and wild animals. Consequently, particular attention should be given to wildlife, as they can directly enter the food chain or come into contact with livestock , acting as a reservoir for transmitting pathogens to humans[27].

Since the 1940s, the use of antibiotics to eliminate pathogenic microorganisms in humans, farm and companion animals and seafood farming has increased dramatically. Although antibiotics have been beneficial to human society in the fight against pathogenic bacteria, saving millions of lives, their use, misuse and abuse has over time resulted in a strong selective pressure in microbial populations, causing the emergence of resistance mechanisms and the spread of antimicrobial-resistant microorganisms (AMR)[28]. The ongoing research and introduction of next generation antibiotics has amplified the selective pressure already present, leading to the emergence of multi-resistant strains. The ability of these antimicrobial resistances to be transferred to other microorganisms, in the environment, in food and in the human gut is a major concern. Adopting One Health approach is crucial to effectively address and combat this ongoing emergency[29,30].

In the current context, the use of advanced technologies is necessary to study microbial heterogeneity along the food chain. In particular, this would allow pathogenic, AMR and spoilage-causing

microorganisms to be identified and characterized in a sensitive, precise, rapid and effective manner, helping to prevent the spread of disease and improving food safety and quality. Finally, the possibility to investigate the mechanisms of antimicrobial resistance and their transmission will reduce the burden on the healthcare system and economic losses related to AMR [31].

2. Application of whole-genome sequencing (WGS) to the risk assessment

Globalisation is revolutionising the way in which food is produced, transported, stored and consumed. As a consequence, food safety systems are subject to continuous changes in terms of regulatory systems, surveillance, coordination mechanisms and emergency responses. Proper risk assessment, prevention of foodborne outbreaks and anticipation of potential emerging risks require advanced monitoring and surveillance systems. The ability to identify and characterise a microorganism, be it pathogenic, causing spoilage, is crucial to ensuring safety and quality of food from field to fork.

Next Generation Sequencing (NGS) technology has revolutionised this field, allowing the entire DNA sequence of micro-organisms to be analysed and thus providing detailed information on genomic composition, biological processes and relationships between the strains[32,33]. Recently, EFSA developed the One Health WGS System, based on the interchange of core genome Multi Locus Sequence Typing (cgMLST) profiles and minimal metadata with the European Centre for Disease Prevention and Control (ECDC) Molecular Typing system, [34,35].

EFSA is currently using the WGS approach for the risk assessment of microorganisms used as novel starter cultures and microbial feed additives. WGS data is used for taxonomic studies, and to investigate the presence of potential AMR genes or genes related to potential pathogenicity. [36]. Earlier traditional molecular techniques, enabling the identification and characterisation of microbial pathogens, were used instead of WGS. These included Pulse-Field Gel Electrophoresis (PFGE), Multi-Locus Sequence Typing (MLST), and Multi-Locus Variable-Number of Tandem Repeats Analysis (MLVA)[37]. These techniques have been crucial earlier for studying food-borne disease outbreaks, as well as assessing microbial relatedness. PFGE was chosen by PulseNet, a national laboratory network that connects foodborne, waterborne, and One Health–related illness cases to detect outbreaks, as the primary method for pathogen strain differentiation due to its high discriminative power, reproducibility and epidemiological consistency[38]. Although these typing techniques have been efficiently used in the studies of food-borne outbreaks for more than 20 years, they are much more laborious and less discrimantive than the WGS approach. The rapid development of NGS and high-throughput sequencing technologies, open sharing of genomic data and open source bioinformatics software tools are leading the way for the use of WGS in food microbiology[33]. Furthermore, the implementation of WGS in food microbiology helps to reduce the food safety risks and economic losses related food recalls[39]. WGS provides not only the ability to reconstruct, but also to detect new food-borne outbreaks rapidly, allowing timely intervention during

food epidemics. Besides supporting the risk assessment, WGS has facilitated the taxonomic reorganisation of several bacterial genera and provided a more accurate assessment of genes contributing to virulence and AMR. It has also provided a better understanding of the transmission of virulence and resistance genes throughout the food chain, leading to an improved overall safety assessment of their host strains[32,40]. Currently WGS is an integral part of EFSA's risk assessment work and improves the response to food emergencies and supports risk management, but, crucially, it also aids in hazard identification and characterisation.

3. WGS supports food safety assessment

3.1. Taxonomic identification of microorganisms

The availability of WGS has revolutionised the taxonomic identification and thus the characterisation of microorganisms in terms of time and accuracy. The conventional polyphasic approach for species identification employed for decades, which encompassed techniques such as 16S rRNA gene Sanger sequencing, DNA-DNA hybridization (DDH), and G+C content determination have now been largely replaced with the WGS approach[41].

Besides typing strains of a specific pathogenic micro-organisms, WGS enables the assessment of taxonomic relationships between different strains, enabling identifying the source of food-borne outbreaks. The evolutionary history of pathogenic strains isolated from infected individuals and their genetic relatedness to other strains of the same species can reveal the source of the food-borne outbreak. It also provides information whether any new pathoghenic strains are circulating in the food chain and/or population [39].

In WGS analysis, the ability to correlate food and patient microbial isolates is achieved through identifying single nucleotide polymorphisms (SNPs) and allele-based differences. Few nucleotide differences between isolates from food and human infections implies relatedness between the isolates, thus indicating the food to be the source of infection. Furthermore, the genetic varibility assessment should also take into account the mutation rate, which characterizes different species; therefore, the exact number of SNPs to define different strains should be determined case by case, in a species-based manner. [42]. Allele-based approaches, such as MLST and MLVA where orthologs are identified using an automated approach against a curated database of possible alleles, are used to confer a sequence type assigned to the isolate that can be used for downstream phylogenetic analyses [43]. Currently, Average Nucleotide Identity (ANI), DNA-DNA Digital Hybridization (dDDH) and Pangenome analysis are the methodologies that refine taxonomy, which is necessary to establish a Overall Genome Relatedness Index (OGRI) with accepted similarity values per species[44].

OGRI defines acceptable threshold values for bacterial species level identification corresponding to similarity values of 96 % for ANI, 70 % for dDDH and 98.65 % for the 16S rRNA gene. For example,

the OGRI approach was used to demonstrate that *Enterococcus faecium* clade B actually belongs to *Enterococcus lactis*[45]. Furthermore, by combining these values with the analysis of homologous genes present in a given dataset (core genome), phylogenetic trees can be delineated based on the nucleotide variations of these genes[46,47]. Using this approach it has been possible to reclassify and determine new genera and species of the *Lactobacillaceae* family[48].

3.2. AMR and mobile genetic elements, the case of nosocomial multi-drug resistant pathogen *Enterococcus faecium*

EFSA and the World Health Organisation (WHO) recommend the use of WGS for safety assessment of microbial strains. In One Health approach to integrate human health, animal health and the environment, WGS is an effective tool to assess the presence of antibiotic resistance genes[35]. The outbreak surveillance system should involve, in addition to taxonomic and epidemiological data, the determination of genomic characteristics such as antimicrobial resistance gene profile and the presence of mobile genetic elements (MGE). Indeed, the advantage of genome analysis is to simultaneously explore the presence of resistance genes and their genomic context, being particularly effective in the study of multidrug resistance (MDR) and point mutations responsible of AMR development.

Several databases such as ResFinder, Comprehensive Antibiotic Resistance Database (CARD), Basic Local Alignment Search Tool (BLAST) , ABRicate, Search Engine for Antimicrobial Resistance (SEAR), ARG-ANNOT and Antimicrobial Resistance Identification By Assembly (ARIBA) are available for the identification of AMR genes at the Center for Genomic Epidemiology (CGE)[50–53]. Antibiotic resistance may be either intrinsic or acquired through point mutations and horizontal gene transfer (HGT) from other strains[54].

Three different mechanisms regulate the HGT of AMR genes between bacteria, transformation (absorption of naked DNA), transduction (transfer via bacteriophages) and conjugation (transfer via plasmids and other MGEs)[55,56]. It is believed that the main mechanism governing the spread and transmission of antimicrobial resistance is conjugation, which can occur in the environment, food and the gastrointestinal tract of humans and animals[57]. Although the data obtained from WGS are not able to assess the frequency of these transfer events or provide information on the AMR gene expression, but only provide information on the presence and absence of AMR genes, the WGS data is essential for mobilome typing. The mobilome typing, which consists of a set of mobile genetic elements that contribute to the variable part of the bacterial genome, typically involves the detection of insertion sequences (IS), pro-phages, integrative and conjugative elements (ICE) and plasmids. The evolution of the bacterial genome and the ability to adapt to adverse conditions are dependent on these regions, which, due to their intrinsic properties, can be shared by different strains of the species conferring new properties. [58]. Conjugative and mobilizable plasmids, independent structures from the bacterial

chromosome, can provide insights to recent gene transfer events and transmission pathways[59]. Obtaining complete plasmid sequences is important because it allows to define the genetic context of specific AMR genes, and to establish whether the plasmid carries the molecular machinery for conjugation and thus to predict the mobility of a plasmid[60]. Several studies have investigated, via WGS, the presence of MDR bacterial strains in ready-to-eat foods such as dairy products and fermented meats, providing information on the spread of AMR along the food chain[61–63]. Of a particular interest has been the isolation of vancomycin-susceptible MDR (VSE-MDR) *E. faecium* strain UC7251 from fermented sausages. Genomic analysis revealed multiple cassettes of antibiotic and heavy metal resistancse on a mobilisable plasmid and a Tn*916* chromosomal transposon in this strain[63].

E. faecium, a lactic acid bacterium ubiquitous in nature, is often detected in fermented foods of animal origin, in which it can take part in the natural fermentation processes. *E. faecium* strains can also be used as starter cultures[64]. More recently, *E. faecium* has emerged as a significant nosocomial pathogen with resistance to multiple drugs. It is accountable for hospital-acquired infections including endocarditis, urinary tract infections, and septicemia[65]. Despite being used as a probiotic and in starter cultures, *E. faecium* lacks QPS status due to its pathogenic potential[14]. EFSA has developed a safety assessment framework for *E. faecium* strains due to its dual nature. This scheme, which focuses on the absence of genetic markers found in hospital-associated (HA) strains, aims to ensure food safety when *E. faecium* strains are used in foods.

E. faecium population is categorized into three main groups: clade A1, which includes HA strains; clade A2, dominated by animal-associated isolates, and clade B characterized by community-associated (CA) strains lacking HA traits[66]. Recently, Belloso Daza and colleagues proposed reclassification of clade B isolates as *Enterococcus lactis* due to their closer genomic proximity to this new species and lack of HA markers[45]. MLST categorizes *E. faecium* into sequence types (STs) based on the allelic profile of six housekeeping genes. The ancestral HA clade A1 is linked to ST17, forming clonal complex 17 (CC17), with the majority of hospital-related isolates falling within CC17[67]. Strain UC7251 is grouped among clade A2 isolates and belongs to ST673 part of CC117, which does not carry virulence factors typical of HA strains but presents the co-location of several antimicrobial resistance genes with heavy metal resistances on the mobilizable plasmid pUC7251_1 and the conjugative transposon Tn*916*[63].

Clade A isolates are commonly resistant to aminoglycosides, ampicillin, and vancomycin and carry different virulence factors and mobile genetic elements that distinguish them from clade B strains[68]. Significant virulence genes in this clade are those encoding surface components crucial for adhesion, biofilm creation, and pili assembly. WGS has enabled the detection of genetic markers contributing to genomic plasticity and additional putative virulence markers (PVM) involved in the colonisation and resistance, especially in HA clade A strains[69]. The emergence of clade A in clinical environments was probably driven by the acquisition of ampicillin resistance due to a mutation in penicillin-binding protein 5 (PBP5)[70]. The evolutionary changes in PBP5 in *E. faecium* closely correspond to the phylogenetic

variation within the species. Clade A isolates with ampicillin resistance possess PBP5-R sequences, while clade B genomes consist of susceptible strains characterized by the PBP5-S profile[71].

The presence of MDR enterococci in the food chain constitutes a significant public health concern, primarily owing to their rapid spread in the environment and between animals and humans. Livestock and agricultural environments represent considerable reservoirs of antibiotic-resistant bacteria due to the extensive prophylactic use of antibiotics, especially in pig meat production[72]. The high number of MDR enterococcal strains isolated from fermented food and their ability to transfer these resistances raise concerns for the safety of products that are not subject to microbial inactivation treatments prior to human consumption [73].

Currently, with the emergence of MDR enterococci and HA isolates, the EFSA safety assessment criteria for *E. faecium* include ampicillin MIC of ≤ 2 mg/L, and the lack of IS*16*/*esp*/*hyl* genes associated with plasticity, adhesion and carbohydrate metabolism respectively[74].

The HGT is a fundamental process in the spread of AMR genes and virulence markers among bacteria, enabling their survival and adjustment in challenging environments. The transfer of AMR genes between *E. faecium* and other bacterial species has primarily been studied in clinical environments; subsequently gene exchange in food matrices has also been verified[75]. Moreover, the transmission of resistance to linezolid[76], oxazolidinone[77], aminoglycosides[78] glycopeptides, erythromycin, and tetracycline[79] has been confirmed among strains isolated from food.

Today, AMR detection has extended to the retail space, where the spread of antimicrobial resistance genes (ARGs) are evident in RTE foods like dairy products, salads, seafood, meat products and pork-derived items[73,80–82].

Although WGS is a powerful tool to study AMR genes and their spread, it is important to remember that databases are not all-encompassing and cannot identify new AMR genes. It is therefore necessary to also perform phenotypic tests, as phenotypic resistance could indicate the presence of yet unknown AMR genes.

3.3. Decoding Virulence in Shiga Toxin-Producing *Escherichia coli* (STEC) through Whole Genome Sequencing

In addition to the detection of AMR genes and MGEs, WGS also allows screening of the known virulence related genes, refining hazard identification and the assessment of potential risks. Genomic data allow the detection and evaluation of virulence gene markers coding for toxins, attack factors, adhesion and invasion factors, immune evasion and replication factors. The databases supporting WGS are based on genome-wide association studies (GWAS). These studies correlate the virulence pattern, at genes level, with the ability to cause disease by testing in cell *in vitro* or *in vivo* models[83,84]. Pathogens

exhibit intra-species variability in theirvirulence genes combinations, and some combinations are considered particularly dangerous for human health[85]. Therefore, not all strains belonging to the same species are able to cause the same diseases, with the same degree of seriousness. It is well known that only a fraction of *Salmonella* strains, as well as *Escherichia coli*, can cause disease in humans[85,86]. Although the presence of virulence genes does not automatically mean that the strain is pathogenic, access to comprehensive data on the presence of virulence factor genes and genes responsible for persistence/survival in food allows a more targeted and effective risk assessment. The virulence factor (VF) search tools, based on databases with homologous genes or proteins previously identified as pathogenicity factors, include VirulenceFinder[87], SPIFinder, Virulence Factor Database (VFDB)[88] and AMRFinderPlus[89]. Furthermore, attention should also be paid to the presence of prophages, temperate phages integrated into the bacterial chromosome, given that they are capable of carrying and transferring genes coding for ARM and toxins[55]. WGS enables the identification and characterisation of phage sequences, making it possible to define the phage type and the potential transmissible genes carried by it[90]. Interesting studies on the distribution of VFs have been done utilising WGS; an example is the surveillance of virulence genes in *E. coli* STEC strains isolated from semi hard raw milk cheese[91,92].

E. coli (STEC) infections in Europe have increased in the last years, being the fourth most reported zoonosis, with 4,824 confirmed cases in 28 European countries in 2020 [93]. Infections attributed to STEC strains result in outbreaks of severe conditions like hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), resulting in a significant public health issue[94].

Currently, serogrouping is one of the criteria used to identify STEC strains with the potential to cause human disease[95]. The "top 5" STEC Serogroups O157, O145, O111, O103, and O26, have been identified as responsible for severe diseases, with O157:H7 recognised as the main cause of severe STEC outbreaks worldwide[96]. The principal virulence factors of STEC are the Shiga toxins (Stx), which contribute to conditions like HC and HUS[97]. Two variations of Stx, namely Stx1 and Stx2, encompassing subtypes Stx1a, c, d, e and Stx2a-i can be distinguished by nucleotide variations and serological reactivity[98]. STEC strains producing Stx1a, Stx2a, and/or Stx2d are linked to the most severe cases of HC and HUS [99]. Lambdoid phages, wich harbor the *stx* genes, remain within the host genetic material until an external signal (for example UV radiation or antibiotics) causes the activation of the lytic cycle and the synthesis of phage particles, allowing horizontal transmission of toxin genes. The *stx1*and *stx2* genes, located downstream of antiterminator Q and upstream of the lysis cassette in the late prophage region, are regulated by the late promoter pR'. The interaction of the Q protein with the Q utilization site (qut), situated on the pR' region, facilitates RNA polymerase to overcome the early terminator cassette, initiating the transcription of the toxin gene[100]. Thus *stx* gene production is activated by phage lysis-inducing agents such as mitomycin C, suggesting that the *stx* gene is transcribed from the PR' of the Stx-encoding phage. Furthermore, present exclusively in stx1-encoding phages, there is a regulation driven by the stx1 promoter (P Stx1). This regulation is represented by the functional operator-binding

site (Fur box) for the Fur, a repressor of iron transport systems in *E. coli*. Consequently, at low iron concentrations, transcription of the *stx* gene occurs without inducing phage lysis[101].

Although the pathogenicity of STEC strains is directly related to the presence of Shiga toxins, the ability to cause disease is also linked to other factors. The pathogenicity island (PAI) of the Locus of Enterocyte Effacement (LEE) harbours the *eae* gene, coding for the intimin protein, which mediates epithelial cell adhesion and toxin release resulting in loss of intestinal microvilli and severe diarrhea, particularly in association with *stx2* gene[102].

Although the most virulent strains are LEE-positive, various non-O157 LEE-negative STEC strains have been identified associated to human disease, indicating their potential to express alternative virulence factors[103,104]. Indeed, they can produce different virulence factors, involved in other adherence mechanisms encoded by plasmids, non-Stx prophages or different PAIs. Three specific PAIs have been documented as being uniquely found in LEE-negative STEC strains. These include the Locus of Proteolysis Activity (LPA), the Subtilase-Encoding Pathogenicity Island (SE-PAI), and the Locus of Adhesion and Autoaggregation (LAA)[105,106]. Databases such as IslandViewer 4 and GIHunter predict the presence of PAI[107]. Recently the LAA PAI, a 86 kb region divided in four modules containing the *hes* gene coding for haemagglutinin, was detected in the *Escherichia coli* O174:H2 strain UC4224 isolated from dairy. This strain harbors both Stx1- and Stx2-bacteriophages[92]. *E. coli* O174 strains have commonly been identified within the top 10 STEC serotypes present in animals, food, and humans[93]. Two novel LAAs and one ICE were recently identified in a genome-wide analysis of 367 LEE-negative STEC strains, including serotype O174, isolated from humans, food and animals [105]. Recent studies provide evidence supporting the involvement of LAAs in the intestinal colonisation of a mouse model and STEC, *stx1-2* genes delete, infection in mice and *Galleria mellonella* larvae[92,105].

Further efforts are needed to improve our understanding of food-derived STECs, in particular to better elucidate the importance of non-stx non-LEE virulence markers in delineating the pathogenic potential of dairy isolates.

3.4. The role of Quorum Sensing System in Biofilm Formation and Stress response

Quorum sensing (QS) mechanisms control microbial proliferation and bacterial communication, having an important role in biofilm formation, and in the expression of virulence factors and stress adaptation mechanisms. The ongoing advancement of high throughput sequencing technologies and the increase in available genomic data allow for an increasingly detailed study of the dynamics of microbial replication, survival and dissemination in the environment[108]. Furthermore, the study and understanding of QS in different bacterial species requires detailed analyses from the information provided by genome sequencing. A recent study, based on WGS, revealed that the QS of *Pseudomonas aeruginosa* PAO1 is regulated either via the normal Rhl system or the transcriptional regulator mexT[109]. The same strain of

P. aeruginosa has been used to assess the ability to attach and create biofilms on different types of microplastics, providing useful information on the role of QS[110]. The ability to produce Extracellular Polymeric Substance (EPS) facilitates bacterial adhesion and attachment, increasing the ability of biofilms to overcome exogenous stresses. An example are the studies conducted on the ability of *Listeria monocytogenes* to form biofilms, in which genome investigations have identified a close correlation of genetic markers inlA, SSi and ermC, to the source of isolation[111,112]. Furthermore, WGS revealed that the surface protein esp, involved in biofilm formation and surface adhesion in *E. faecalis* and *E. faecium* is within a PAI. It was also demonstrated that only *E. faecium* isolates from clinical infections contain PAI with esp[69].

Bacteria are often subject to disadvantageous environmental conditions under which they need to adapt to survive. These adverse conditions, perceived as stress, induce intricate stress response systems especially in pathogenic microorganisms. A study by Liu and colleagues used NGS data to investigate the oxidative stress response of *Salmonella* ser. Enteritidis. Their findings suggest that there is a relation between virulence and oxidative stress. In particular oxidatively stressed *S. Enteritidis* cells simultaneously repressed key motility encoding genes and induced a wide range of adhesin- and salmonellae-essential virulence-encoding genes, that are critical for the biofilm formation and intracellular survival[113]. The stress response involves various molecular pathways at the level of transcription, translation and proteins. Understanding the mechanisms of stress response and adaptation to the environment of pathogenic micro-organisms in the food chain would facilitate the development of new strategies to control these micro-organisms that pose a risk to human health[114,115].

Finally, susceptibility to antibiotics has been demonstrated to be reduced by the activation of stress responses, through the stimulation of resistance mechanisms, the promotion of biofilm formation and the induction of mutations leading to resistance. Thus, the activation of bacterial stress responses poses a threat to the efficacy and clinical outcome of antibiotic therapy. However, stress responses in bacteria may also prove to be potential targets for therapeutic alternatives to antibiotics[116].

4. *In vivo,* food and environmental models to study HGT (Horizontal Gene Transfer) and VF (virulence factors)

WGS has revolutionized the understanding of AMR in terms of ARG identification and emerging resistance detection, tracking transmission, resistome analysis and epidemiological studies. While WGS has changed the field of AMR research, it also presents challenges related to the inability to obtain a minimum inhibitory concentration (MIC) or inhibition zone, and it also does not provide direct information on levels of gene transmission in term of conjugation frequency. Similarly, the identification of virulence factors via WGS requires correlation with pathogenicity studies in *in vitro* or *in vivo* models. Consequently, the genotypic approach needs to be complemented by *in vitro* and *in vivo* studies.

Current insights into the dissemination of antibiotic resistance have mainly been obtained from *in vitro* or observational studies. Nevertheless, *in vitro* knowledge on predicting the horizontal transfer of ARG remains limited. Furthermore, *in vitro* models may not accurately replicate the HGT of resistance genes *in vivo*[117]. Thus, it is imperative to employ models that faithfully replicate natural contexts observed in foods, humans, animals and the environment to complement the present understanding of ARG transfer in bacterial populations.

Food models are essential tools for studying ARG transfer among bacteria in the food chain. These models aim to replicate real-world conditions within the food supply system on a laboratory scale, enabling the investigation of the dissemination dynamics of ARG. For example, some studies have investigated the transfer of ARG modelling the ripening of fermented meat and cheese [118–120]. Interestingly, other studies have performed *in situ* conjugation experiments in salmon and fermented chicken sausage, where Tn*916*-carrying *tet*(M) was transferred from *L. salivarius* and *L. monocytogenes* to *E. faecalis* [121,122]. Other studies have also explored the capacity of Tn*916*-mediated transfer of *tet*(M) from *L. monocytogenes* to *E. faecalis in situ* on the surface of cheese, with results comparable to ours [123,124].

Furthermore, the use of food models is essential to evaluate the persistence and replication capacity of pathogenic microorganisms during food processing. For example, using a laboratory-scale model it was possible to demonstrate that the process of making a semi-hard raw milk cheese was not able to prevent the growth of *E. coli* STEC[92]. Similar study confirmed that the production processes of variety of soft and semi-hard raw milk cheeses were not effective in controlling the growth of STEC strains[125–129].

Foods are an excellent carrier for the spread of AMR and pathogenic bacteria in the gastrointestinal tract of humans and animals. It is therefore essential to employ *in vivo* models that can closely reproduce the conditions present in human and animal gut. Various animal models, including those for insects, mice and aquatic organisms, are currently used to directly observe the interactions between pathogens and their host organisms, as well as the gene transfer capacity between resident and transient bacteria in the gastrointestinal tract[130,131]. Insects larvae, such as *Galleria mellonella,* have become the ideal surrogate host organism for gene transfer and virulence studies due to the presence of mammalian-like innate immune system and easiness, rapidness and low costs related tothe use of this insect model, s[132]. For example, Göttig and colleagues investigated the HGT of gene OXA-48 using *G. mellonella*, obtaining more realistic results than those obtained *in vitro*[133]. Another study demonstrated the capability of S*taphylococcus aureus* to disseminate a gentamicin resistant plasmid in the larvae of the coleoptera *Tenebrio molitor* (mealworm)[134]. Yet another study shows that larvae of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) support HGT of AMR genes between *Salmonella enterica* and *Escherichia coli* and that the exchange may occur within their gastrointestinal tract under laboratory conditions[135].

In addition to being a valuable tool for HGT studies, the insect larvae are a useful model to evaluate the *in vivo* bacterial pathogenicity and to elucidate the individual contributions of various virulence markers. Numerous studies have investigated the pathogenicity of STEC, enteropathogenic (EPEC) and

Enteroaggregative (EAEC) *E. coli* using *Galleria* larvae as the host. For example Ikeda and colleagues assessed the virulence of different bovine *E. coli* STEC and EPEC O80:H2 strains and the roles in pathogenicity of pS88 plasmid and Shiga toxin 2d using *Galleria* larvae[131]. Another study using *Galleria* larvae investigated ithe virulence of 6 EAEC strains, harboring the transcriptional factor AggR, the aii island encoding a type VI secretion system, the dispersin and the dispersin transporter[136].

Milani et al. 2023 evaluated the role of shiga toxin stx1a and stx2a in *G. mellonella* infection, creating STEC mutants lacking one or both the toxin encoding genes. They found that virulence was related not only to *stx* genes but to other virulence factors as well[92]. Furthermore, Habets and colleagues assessed the pathogenicity of Stx2d phage transduced from a STEC O80:H2 strain to a non-STEC strains, also in *Galleria* larvae[137].

Employing larvae as model hosts offers a multifaceted approach to unravel the intricate dynamics of bacterial pathogenicity and transferability of AMR, providing insights into the avenues for developing targeted interventions for combatting both bacterial infections and the spread of AMR in humans and animals.

Currently the poorly controlled use of antimicrobial agents in aquaculture has significantly contributed to the increase of AMR within the marine ecosystem. The spread of AMR in marine ecosystems is now a growing concern due to its potential impact on aquatic life, human health and global ecosystems. Marine environment is considered to be a reservoir for various microorganisms, including bacteria carrying ARGs[138]. Marine animals, including fish, molluscs, and crustaceans, can harbour AMR bacteria due to the exposure to contaminated water and feed, acting as potential carriers of resistant bacteria to humans through seafood consumption[139]. Particularly, mussels are considered a useful bioindicator of water contamination and they are used in monitoring the presence and diffusion of ARGs in marine environments. Due to their filtration behaviour, mussels can accumulate a wide range of microorganisms from the surrounding water, providing a model for assessing HGT. Previous studies support the fact that ARG transfer can occur in the aquatic ecosystem and in marine mussels acting as a reservoir of AMR[140–143].

Adding complexity to the scenario, bacteria frequently create biofilms on various surfaces including microplastics, emerging pollutants in the marine environment. These biofilms serve as reservoirs for ARG, enabling their transmission to other bacteria within the biofilm and upon their release into the water column[144]. The role of microplastics as a vehicle for the spread of ARG in aquatic environment has been observed in different studies[145,146]. Furthermore, additional research provides support for the role of microplastics in facilitating the development of biofilms, thereby substantially enhancing the transfer of ARGs compared to water samples lacking microplastics[147–149]. Indeed, Arias-Andres and colleagues observed a notably elevated rate of plasmid transfer among microplastics-associated bacteria in comparison to bacteria existing freely or within natural aggregates in aquatic setting[150,151].

Food and *in vivo* models offer controlled environments to manipulate various test parameters and test hypotheses related to the transfer of AMR. Moreover, these models offer a powerful tool for assessing the pathogenicity potential and the persistence of pathogenic microorganisms to food processes. However, they also bring challenges, including the complexity of replicating real-world conditions, the need for careful validation, and the potential to oversimplify complex processes. To maximize the relevance of the findings, researchers often combine data from various models and integrate them with real-world surveillance and clinical data. This holistic approach helps us to understand the role of the food supply chain and devise strategies to mitigate its impact on public health.

5. Mitigation and preservation: LAB as guardians of Food Safety and Security.

The primary challenge of the food industry is to ensure food safety. Therefore, efforts to prevent this challenge should be based on the development of risk mitigation strategies to counter the increased spread of AMR, pathogenic and spoilage-causing bacteria during food production, storage, and distribution. The application of Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP) and Good Hygienic Practices (GHP) together with the information gathered from surveillance are crucial for effective food safety management[152].

Efficient strategies for preventing the transmission of AMR bacteria in food of animal origin include conservative use of antimicrobials in animal husbandry and implementation of control procedures for all foods during processing [153]. Guaranteeing the safety of fresh and fermented products is a significant challenge for food industry, as physical processing methods alone are often not sufficient to guarantee the consistent production of safe food[154]. In fact, microbial contamination of fresh and processed meat products by various pathogenic and spoilage microorganisms is a major concern. In recent decades, these problems have encouraged researchers to develop new control measures, such as bioprotectors and protective food cultures. Given their generally recognised as safe (GRAS) status the use of LAB as biocontrol agents, in addition to their role as starter cultures, has been proposed as a sustainable alternative to antimicrobials[155].

The primary antimicrobial effect of LAB derives from the decrease of pH in food and the activity of organic acids. Some LAB also produce bacteriocins and bacteriocin-like inhibitory substances (BLISs). Hence, incorporating LAB, with the capacity to produce antimicrobial compounds, in food during the manufacturing process can counteract the growth of pathogenic and spoilage microrganisms. Several species of LAB, including *Latilactobacillus sakei*, *Lactiplantibacillus plantarum*, *Ligilactobacillus animalis* and *Latilactobacillus curvatus*, are used as bioprotective agents in meat products. Their antimicrobial activity, provided by metabolites such as organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins, has been extensively studied in food matrices against the growth of pathogenic or spoilage microorganisms [156–163].

Among the metabolites of LAB, bacteriocins such as nisin, enterocin, plantain, pediocin, pentocin, pneumocyclin, curvacin and sakacin, are of particular interest for the dairy and meat industries. These small antimicrobial peptides have been shown to be quite effective against food pathogens such as *E. coli* STEC, *L. monocytogens, Staphylococcus aureus,* and *Salmonella* sp., as well as spoilage microorganisms[10,163].. Bacteriocins are generally classified based on their bacterial source (from both Gram-positive and Gram-negative bacteria), molecular size, heat stability, chemical structure, biochemical properties and mode of action[164]. Bacteriocins obtained from the LAB can be divided into three categories: class I, class II and class III. Class I bacteriocins, also called lantibiotics, are thermostable peptides less than 5 kDa in size, synthesized with the amino acids lanthionine and/or methyllanthionine in the ribosome. Lantibiotics are further divided into different subclasses (AI, AII and B), based on their structural and functional differences. The peptides of subclass AI are elongated, positively charged and act by forming pores in the cytoplasmic membranes, while those belonging to subclass B are globular in shape, negatively or neutrally charged and act through cell wall biosynthesis mechanisms[164].

Class II bacteriocins, differently from class I bacteriocins, are hydrophobic peptides that exhibit no post-translational modification. This class of bacteriocins, which are thermostable and of low molecular weight (<10 kDa), have an amphiphilic helical structure, which depolarises the bacterial membrane, leading to the death of pathogens. These non-lantibiotic bacteriocins are subdivided into subclasses IIa, IIb, IIc or IId based on their N-terminal amino acid sequences. Subclass IIa bacteriocins, such as pediocin PA-1, leucocin A, sakacin A, P, T, Q, X, G and enterocin A, are very specific against *Listeria* spp. and contain one or two α-helices in the N-terminal peptide chain. Recently, 28 class IIa-like bacteriocins with high specificity against *L. monocytogenes* called 'pediocin boxes' were identified [165]. The bacteriocins of subclass IIb are heterodimeric and therefore require the synergistic action of two peptides to permeabilise the target bacterial membrane and decrease the intracellular ATP concentration. Lactocin 705 produced by *L. curvatus* CRL705, plantaricin from *L. plantarum*, enterocin from *E. faecalis* and lactococcin from *L. lactis* are part of subclass IIb Subclass IIc bacteriocins, which are characterised by a cyclic structure, include e.g. circolarin A from *Clostridium beijerinckii*, reutericin 6 from *Limosilactobacillus reuteri*, enterocin AS-48 from *E. faecalis*, carnocycline A from *Carnobacterium* spp. and garvicin ML from *Lactococcus garvieae*[166]. Finally, unmodified, linear bacteriocins and non-pediocins belonging to class IId, which currently includes 31 types mainly produced by LAB, such as lactococcin[167].

The class III category of bacteriocins produced by LAB are thermolabile macromolecules with a particle size greater than 30 kDa. This class includes subclass IIIa or bacterolysin, which includes lysostaphin and enterolysin A that are active on the cell wall, and subclass IIIb, which includes elveticin M that dissipates the membrane potential and reduces the intracellular ATP concentration[168].

Recently the interest in traditional and regional food products has increased. This can pose a challenge for the food industry, which has to meet the market demands for producing diversity of food products[169,170]. The use of indigenous starter cultures (i.e. cultures originally isolated from naturally

fermented products) can help to produce traditional foods that are not only safe but also of exceptional quality. Moreover, it has been established that indigenous starter cultures often improve the sensory characteristics (taste, texture, colour) of fermented products,[171]. As a result, the food industry is actively seeking indigenous LAB bacteria possessing both technological and antimicrobial properties to serve as starters and protective cultures in meat products. LAB strains potentially used as starter cultures must be safe, grow at different temperatures and in different salinities and allow for rapid pH decline in the food matrix, as well as produce a satisfactory flavour profile typical for the product[172,173].

Several studies have evaluated the contribution of indigenous LAB strains, belonging to the species *L. sakei*, *L. plantarum* and *L. curvatus,* to the fermentation of meat products resulting in the formation of a characteristic sensory profile of the product[174–177]. The species *Companilactobacillus alimentarius* is often detected as part of the microbiota of traditional fermented foods, in which it plays crucial functions in the fermentation and maturation processes. They are also recognised as QPS and are part of the inventory of food microbial cultures with demonstration of safety in fermented foodss[48,176,178].

The versatility of LAB in improving safety, flavor and shelf life offers a promising future for their use in the meat industry. Since consumer demand for naturally preserved, flavor rich and safe meat products continues to increase, The role of LAB as champions of high quality and traditional foods will grow. Their contribution to the production of safe and high-quality meat products involves continuous search for new species and new indigenous strains to be used as protective or starter cultures.

6. References

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LIST OF ABBREVIATIONS

(AMR) Antimicrobial-resistant

(ARGs) Antimicrobial Resistance Genes

(ARIBA) Antimicrobial Resistance Identification By Assembly

(BLAST) Basic Local Alignment Search Tool

(BLISs) Bacteriocin-like inhibitory substances

(CGE) Center for Genomic Epidemiology

(CC) Clonal Complex

(CARD) Comprehensive Antibiotic Resistance Database

(CA) Community-Associated

(DDH) DNA-DNA hybridization

(EAEC) Enteroaggregative

(ECDC) European Centre for Disease Control

(EFSA) European Food Safety Authority

(EHEC) Enterohaemorrhagic *Escherichia coli*

(EPEC) Enteropathogenic

(EPS) Extracellular Polymeric Substance

(GRAS) Generally recognised as safe

(GAP) Good Agricultural Practices

(GHP) Good Hygienic Practices

(GMP) Good Manufacturing Practices

(HACCP) Hazard Analysis and Critical Control Point

(HC) Hemorrhagic Colitis

(HUS) Hemolytic Uremic Syndrome

(HA) Hospital-Associated

(HGT) Horizontal Gene Transfer

(IS) Insertion sequences

(ICE) Integrative and Conjugative Elements

(LAB) Lactic acid bacteria

(LAA) Locus of Adhesion and Autoaggregation

(LEE) Locus of Enterocyte Effacement

(LPA) Locus of Proteolysis Activity

(MRSA) Methicillin-resistant *Staphylococcus aureus*

(MGE) Mobile genetic elements

(MDR) Multidrug Resistance

(MLST) Multi-Locus Sequence Typing

(MLVA) Multi-Locus Variable-Number of Tandem Repeats Analysis

(NGS) Next Generation Sequencing

(PAI) Pathogenicity Island

(PBP5) Penicillin-Binding Protein 5

(PFGE) Pulse-Field Gel Electrophoresis

(PVM) Putative Virulence Markers

(QPS) Qualified Presumption of Safety

(QS) Quorum sensing

(SEAR) Search Engine for Antimicrobial Resistance

(STs) Sequence types

(STEC) Shiga Toxin-Producing *Escherichia coli*

(SNPs) Single nucleotide polymorphisms

(SE-PAI) Subtilase-Encoding Pathogenicity Island

(VSE-MDR) Vancomycin-Susceptible MDR

(VF) Virulence Factor

(VFDB) Virulence Factor Database

(WHO) World Health Organisation

CHAPTER 2

Genomic insight of *Enterococcus faecium* UC7251, a multi-drug resistant strain from ready-to-eat foods, highlights the risk of antimicrobial resistance in the food chain.

Genomic insight of *Enterococcus faecium* UC7251, a multi-drug resistant strain from ready-to-eat foods, highlights the risk of antimicrobial resistance in the food chain.

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1. ABSTRACT

The presence of multi-drug resistant (MDR) bacteria in ready-to-eat foods comprises a threat for the public health due to their ability to acquire and transfer antibiotic-resistant determinants that could settle in the microbiome of the human digestive tract. In this study, *Enterococcus faecium* UC7251 isolated from a fermented dry sausage was characterized phenotypically and genotypically to hold resistance to multiple antibiotics including aminoglycosides, macrolides, β-lactams and tetracyclines. We furtherly investigated this strain following a hybrid sequencing and assembly approach (short and long reads) and determined the presence of various mobile genetic elements (MGE) responsible of horizontal gene transfer (HGT). On the chromosome of UC7251, we found one Integrative Conjugative Element (ICE) and a conjugative transposon Tn*916* carrying tetracycline resistance. UC7251 carries two plasmids, one small plasmid harboring a rolling circle replication and one MDR megaplasmid. The latter was identified as mobilizable and containing a putative integrative conjugative element-like region, prophage sequences, insertion sequences, heavy-metal resistance genes and several antimicrobial resistance (AMR) genes confirming the phenotypic resistance characteristics. The transmissibility potential of AMR markers was observed through mating experiments where Tn*916*-carried tetracycline resistance was transferred at intra- and inter-species level. This work highlights the significance of constant monitoring of products of animal origin, especially RTE foodstuffs, to stimulate the development of novel strategies in the race for constraining the spread of antibiotic resistance.

2. INTRODUCTION

Enterococcus faecium is an ubiquitous species found in a large number of foods, mainly fermented products of animal origin like cheeses and fermented sausages (1). Some strains of this species have been also recognized as probiotics conferring benefits to their hosts (2). Nevertheless, in the past three decades, *E. faecium* emerged as an important nosocomial multi-drug resistant (MDR) pathogen responsible for hospital-acquired infections (3). The duality of this species has led the European Food Safety Authority (EFSA) to state a safety assessment scheme based on the absence of genetic markers generally present in the hospital-associated (HA) biotypes for those *E. faecium* strains that are intentionally introduced into the food chain (4). Although *E. faecium* is extensively used as a probiotic and as part of the fermentation processes, it does not actually hold the Qualified Presumption of Safety (QPS) status due to its potential pathogenicity (5).

Previous studies indicated that the population structure of *E. faecium* is divided into three distinct clades. Clade A1 bearing clinical isolates, clade A2 mainly represented by strains from animal and human commensals that might cause sporadic human infections, both carrying determinants for virulence and antimicrobial resistance (AMR). The third, clade B, is characterized by community associated (CA) isolates lacking HA traits (6). Recently, clade B isolates were proposed to be reclassified as *Enterococcus lactis* because of the closer genomic proximity to this new species and lack of HA markers (7). The genetic

transmission of HA markers among isolates, for instance between farm animals and humans in the agricultural setting, revealed consequently the contamination of products of animal origin that affect the entire production and supply chain (8). The rise of MDR enterococci in the food chain represents a major public health concern as they are easily disseminated through the environment (9). Livestock animals and the farm environment exemplify an important reservoir of AMR bacteria due to the widely use of antibiotics (10), particularly in swine for prophylactic reasons (11). Also, resistance to heavy metals is a matter of concern because of possible co-selection of antibiotic resistance. Specifically, resistance towards copper is common in swine derived isolates due to the use of copper sulfate as a growth promoter in feed for pigs (12). Enterococci harboring MDR genes have been frequently isolated from the swine samples (13) and their diffusion arises concerns about the potential transmission to meat-based ready-to eat (RTE) foods, which proposes a risk because of the lack of microbial inactivation prior consumption (14). Considering the emergence of MDR enterococci and HA isolates, the current criteria for safety assessment is represented by a MIC of ampicillin of ≤ 2mg/L and lack of IS16/esp/hyl genes, associated with plasticity, adhesion, and carbohydrate metabolism, respectively (4). Further information on epidemiology and population structure can be analyzed by applying the Multi Locus Sequence Typing (MLST) scheme. Following this, *E. faecium* can be classified in different sequence types (STs), where ST17 was identified as the ancestral clone of HA isolates, forming the Clonal Complex 17 (CC17) (15). Nonetheless, it is crucial to understand the distribution of other putative virulence markers (PVM), involved in colonization and resistance recognized in other studies (3, 16). Horizontal gene transfer (HGT) is one the mechanism at the base of AMR and virulence markers dissemination among bacteria that, facilitates their survival and adaptation in stressful conditions. HGT of AMR genes between *E. faecium* and other species has been investigated mostly in clinical settings; furthermore, gene exchange in food was also demonstrated (14). Additionally, the transfer of resistance towards linezolid (17), oxazolinodone (18), aminoglycosides (19), glycopeptides, erythromycin and tetracycline (20) has been demonstrated between food isolated strains. The detection of AMR has also reached the retail level with the presence of AMR dissemination in RTE foods such as dairy products (21), salads (22), seafood (23) and meat products (24), pork-origin included (25).

Whole Genome Sequencing (WGS) has facilitated the understanding of the mechanisms that support the dissemination of mobile genetic elements (MGEs) in bacteria. The aim of this study is to investigate the genomic characteristics of a Vancomycin-susceptible MDR (VSE-MDR) *E. faecium* strain isolated from ready-to-eat fermented sausage and to evaluate the potential transmissibility of AMR markers through MGEs.

3. MATERIALS AND METHODS

3.1 Bacterial strain, cultivation, and antibiotic susceptibility testing

The strain UC7251 was isolated from a dry fermented Italian salami on Slanetz & Bartley Medium (Oxoid) containing 4 μg/ml Ampicillin (Sigma). The strain was sub-cultivated in Brain Heart Infusion (Oxoid) overnight at 37°C and species-specific PCR using primers for the ddl gene (Table 1S) was performed to confirm its taxonomical classification. Susceptibility to different antibiotics was determined by broth microdilution method according to EUCAST (26). The antimicrobial agents used were ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tylosine, tetracycline and chloramphenicol. The antibiotics were obtained from Sigma (St. Louis, Missouri, USA). The minimum inhibitory concentrations (MICs) were compared to the breakpoints recommended by EUCAST (2003) (http://www.eucast.org/) and EFSA (4).

3.2 Heavy metal susceptibility testing

Susceptibility towards Copper (Cu), Zinc (Zn), Cadmium (Cd) and Mercury (Hg) was tested as previously described (27, 28). Briefly, overnight cultures were spotted onto Mueller-Hinton agar (Oxoid) supplemented with different concentrations (0.05 to 40 mM) of $ZnCl_2$ (Carlo Erba), $HgCl_2$ (Sigma Aldrich) and $CdSO_4$ (Sigma Aldrich) resuspended in distilled water, and CuSO4 (Merck Millipore) adjusted to pH 7.2 with 1 M NaOH. After 24 to 48 h of incubation at 37°C, the plates were visually inspected for bacterial growth on the spots.

3.3 Conjugal Transfer

In vitro conjugation experiments were performed as described before (29). UC7251 was used as donor strain and 29 bacterial strains as recipients (see Table 3S). Briefly, 1 ml of a culture (OD600= 0.8) of donor and recipient strains were passed through a 0.45μm filter (MF-Millipore Membrane Filters, Merck). Right after, the filter was placed onto a non-selective agar plates favoring the growth of recipient strains and incubated at 37ºC for 24h. Conjugation with *Bacillus, Enterococcus, Listeria, Pseudomonas,* and *Staphylococcus* as recipient strains, were carried out onto BHI (Oxoid), *Clostridium* on RCM (Oxoid), for lactobacilli, *Pediococcus* and *Weisella* onto MRS (Difco). After the respective incubation period, cells were resuspended from the filter using saline solution and were diluted in a 10-fold dilution series and enumerated by spread plating onto appropriate agar media. Transconjugant selection was performed using the selective conditions reported in Table 3S. Transconjugant colonies were randomly selected and analyzed to check the presence or absence of the antibiotic resistance genes, by extracting the DNA with microLYSIS kit (Microzone) and performing PCR with primers for tetracycline and erythromycin resistance genes (Table 1S). The passage of potential plasmid-borne antibiotic resistance genes coding

for aminoglycosides (aad6, aph3-IIIa, aadE, satA, ant(6)-Ia) and lincosamides (IsaE, LnuB) resistance, was also tested by PCR using the primers listed on Table 1S.

3.4 Multilocus sequence typing (MLST) analysis

Allelic profiles and sequence types were derived by PubMLST (30). The obtained ST were analysed using Phyloviz and the goeBURST algorithm to compute a spanning forest graph to build the relatedness between isolates based on Single Locus Variants (SLV) to identify clonal complexes (31). Furthermore, given that the resolution of MLST is limited, cgMLST (core genome MLST) was also determined using the cgmlst.org website. This method uses an allele numbering system for a scheme of 1423 cgMLST target genes, which confers a higher level of discrimination (32).

3.5 Detection of markers relevant for the assessment of safety and antibiotic resistance determinants

The strain UC7251 was screened for the hospital-associated genetic markers IS16, hylEfm and esp by PCR, using primers previously listed in the related section. Strains U0317 and E980 were used respectively as positive and negative controls. The presence of the antibiotic resistance determinants coding for the phenotypical resistances observed in UC7251 was investigated by PCR using the primers reported in Table 1S. The complete pbp5 gene was amplified, sequenced, and analyzed as described before (33), while the amplification of ermB, tetM, tetL, aph3-IIIa ,satA ,ant(6)-Ia and aadE was performed as described elsewhere (34–39). Here, new primers aad6_F and aad6_R for aad6 screening, Lnu-B_F and Lnu-B_R for Lnu(B) screening, IsaE_F and lsaE_R for Isa(E) screening were designed de novo using Primer3 (40), and run the amplification reaction with the following conditions: initial denaturation at 95°C for 2 min; 35 cycles at 94°C for 40 s, 53°C for 45 s, and 72°C for 50 s; and extension at 72°C for 5 min.

3.6 Genome sequencing and database submission

A hybrid sequencing approach (short and long read) was followed to complete the assembly of UC7251. Genomic DNA was extracted from the cultured bacterium with NucleoSpin Tissue (Macherey-Nagel, Germany). Short read resequencing was performed with Illumina Miseq, 250 paired-end after Nextera XT paired-end library preparation. Long read sequencing was performed with PacBio Sequel II SMRT sequencing. After trimming the sequences using trimgalore! (GitHub - FelixKrueger/TrimGalore), hybrid assembly was carried out using Unicycler (41).
The finished genome was deposited on NCBI under assembly accession No. ASM41165v2.

3.7 Bioinformatic Analyses

A total of 74 *E. faecium* complete genomes, including reference strains were selected to carry out phylogenetic and taxonomic analyses in comparison with UC7251 (Table 2S). Assembled genomes were downloaded from NCBI in September 2021 and were subsequently annotated using Prokka (42). Annotation results were then submitted to pan- and core-genome analysis using Roary (43). The phylogenetic tree was constructed using RAxML-NG, V1.0.0(44) and iTOL was used to visualize and organize the tree (45). The genomes were also submitted to digital DNA-DNA hybridization (dDDH) using the Genome-to-Genome Distance Calculator (GGDC) (46). Average Nucleotide Identity (ANI) analysis was performed using fastANI (47).

In silico investigation of UC7251 was performed using the bioinformatics software platform Geneious prime v. 10.1. The Basic Local Alignment Tool (BLAST) from NCBI was used to investigate the presence and identity of different genetic markers contributing AMR, VF and MGE. The genome was interrogated for the presence of AMR genes using the Comprehensive Antibiotic Resistance Database (CARD) (48) and ResFinder (49). Ampicillin resistance was studied by evaluating the allelic variation in the strain of interest, against the reference sequence for PBP5-S/R profiles. Virulence markers were investigated according to the latest guidelines of EFSA (4) using manual annotation, VirulenceFinder (50) and VFAnalyzer (51).

HGT determinants were analyzed through MobileElementFinder (52) and Island Viewer 4 (53). In addition, integrative and conjugative elements were predicted using ICEberg 2.0 (54), which detects the signature sequences of the integrative modules and conjugation modules based on the profile hidden Markov models (profile HMMs). The origin of transfer site (oriT) was determined with OriTFinder (55). Lastly, the genome was screened for the presence of sequences of phage origin with Prophage Hunter (56) and CRISPR-Cas sites using CRISPR-CasFinder (57).

4. RESULTS AND DISCUSSION

4.1 Isolation and characterization of MDR *E. faecium* UC7251 from RTE food

In the framework of risk assessment of MDR in ready to eat foods, UC7251 was isolated from a dry-fermented sausage at a count of 3 x 10^5 CFU g-1 and identified as *Enterococcus faecium* by species-specific amplification of the ddl gene. This strain was resistant to ampicillin, streptomycin, kanamycin, erythromycin, clindamycin, tylosine and tetracycline and presented a MIC higher than the cutoff values defined by EUCAST and EFSA (Table 1). PCR analyses, using a pool of primers pairs targeted to the most commonly AMR genes found in enterococci (Table 1S) identified the genetic determinants for these resistances. *E. faecium* UC7251 was identified as a MDR strain, and harbored genes coding for aminoglycoside modifying enzymes, three genes for macrolide resistance and two genes responsible for

tetracycline resistance. Moreover, the sequence of the amplicon targeted to the penicillin binding protein 5 PBP5, involved in β-lactams resistance, demonstrated that this strain showed the pbp5S1/R20 allelic profile, conferring resistance to ampicillin (58). *E. faecium* showed to be intrinsically resistant to low levels of ampicillin through cell wall synthesis protein complex PBP; pbp5 is part of this operon and sequence variations allow to differentiate the two groups of *E. faecium* according to allelic profile and expression levels
(33). Within the context of a study focusing on the detection of ampicillin resistant *E. faecium* in ready to eat fermented foods, a strain that presented resistance towards ampicillin with a MIC value of 64 μg/ml and carried the hybrid allelic profile PBP5-S1/R20 is of concern for the consumers safety. It has been demonstrated that pbp5 may spread through horizontal gene transfer and specifically that pbp5 of resistant isolates was located on transferable chromosomal regions, which suggested its dissemination through the environment (59).

Table 1*. Antimicrobial resistance genes and MIC values of strain UC7251, following the guidelines and cutoff values established by EFSA/EUCAST for the safety assessment of E. faecium.*

Antibiotic Resistance	UC7251 (μg/ml)	EFSA Cut-off value (μg/ml)	EUCAST (μg/ml)	AMR gene
Ampicillin	64	2	4	pbp5-S_1/R_{20}
Vancomycin	1	4	4	-
Gentamycin	32	32	32	*aac(6')-Ii*
Kanamycin	>4096	1024	-	*aph(3')-III*
Streptomycin	>1024	128	128	*aad6, aadE*
Erythromycin	>512	4	4	*erm*B, *mrs*C, *sat*4
Clindamycin	>512	4	-	*erm*B, *Inu*B, *Isa*E
Tylosine	>512	4	-	*erm*B
Tetracycline	128	4	4	*tet*L, *tet*M
Chloramphenicol	8	16	32	-

4.2. Whole Genome Sequence Analyses

UC7251 was submitted to genome sequencing following a hybrid approach using long and short read technology (GenBank assembly accession numbers for chromosome CP084886.1, plasmid pUC7251_1 CP084887.1, plasmid pUC7251_2 CP084888.1). The assembly of the genome of UC7251 built a total of 3 contigs, predicted as a 2,6 Mb chromosome and two plasmids, pUC7251_1 and UC7251_2 (192 kb and 1,9 kb, respectively). The presence of the two plasmids was also distinguished by total DNA extraction and Pulsed Field Gel Electrophoresis (PFGE) (data not shown). The annotation of UC7251 resulted in 2662 coding sequences (CDS), of which 27% are hypothetical proteins and 73% have known functional assignments. It also contained genes coding for 18 rRNAs (6 copies each of 23S rRNA, 16S rRNA, and 5s rRNA), 69 tRNAs and 1 tmRNA. Compositional analysis resulted in 17 genomic islands (GIs), three active prophage sequences and several VF and AMR genes distributed throughout the chromosome and plasmidome. Regarding mobile genetic elements, two mobile regions were predicted on the chromosome and one on pUC7251_1 (Table 4S).

pUC7251_1 is a mobilizable megaplasmid as predicted by Plascad. According to OriTfinder, the origin of replication is 39bp long and showed homology with oriT_pUB110.There are no predicted T4SS proteins and only one T4CP protein on locustag UC7251_02595. The relaxase MobM is found on locustag UC7251_02679. Mobilizable plasmids carry their own oriT and relaxase gene but lack genes required for T4SS formation and can therefore be transferred to cells that carry elements encoding a compatible T4SS (60). This plasmid showed homology with plasmids pF88_1 (identity 83%), p17-318_1 (identity 83%) pE843-TC-299 (identity 82%) and pE843-171 (identity 80%). The first three are VSE-MDR plasmids carried by *E. faecium* strains of clade A2. These strains were isolated from environmental (pF88_1) and human samples (p17-318_1 and pE843-TC-299). The fourth plasmid pE843-171, is carried by *Enterococcus lactis* E843 and it is characterized as VSE-MDR (61). According to these results, pUC7251_1 holds unique traits, and although the prevalence of VSE-MDR is high, none of the results on BLAST showed VSE-MDR from food origin. UC7251_2 harbors a single open reading frame that codes for a rolling circle REP (rep14a). Small plasmid of such size was also found in other *E. faecium* isolates, making it a common genomic feature.

4.3. Phylogenomics and Population Structure show that foodborne UC7251 is neighboring HA isolates.

For phylogenomic evaluation, UC7251 was compared with the other selected 74 *E. faecium* genomes (fig. 1). The interrogation of the pangenome has been recently regarded as a useful tool for species delimitation based on identification of lineage- specific gene sets (62). Observing the distribution of core- and accessory- genomes of our analysis, isolates of clade A1 and to a smaller extent clade A2 have a high variability in their accessory genes. A highly variable accessory genome is conferred by the fact that *E. faecium* has an open pangenome and therefore a higher genomic diversity (6). The adaptation of *E. faecium*

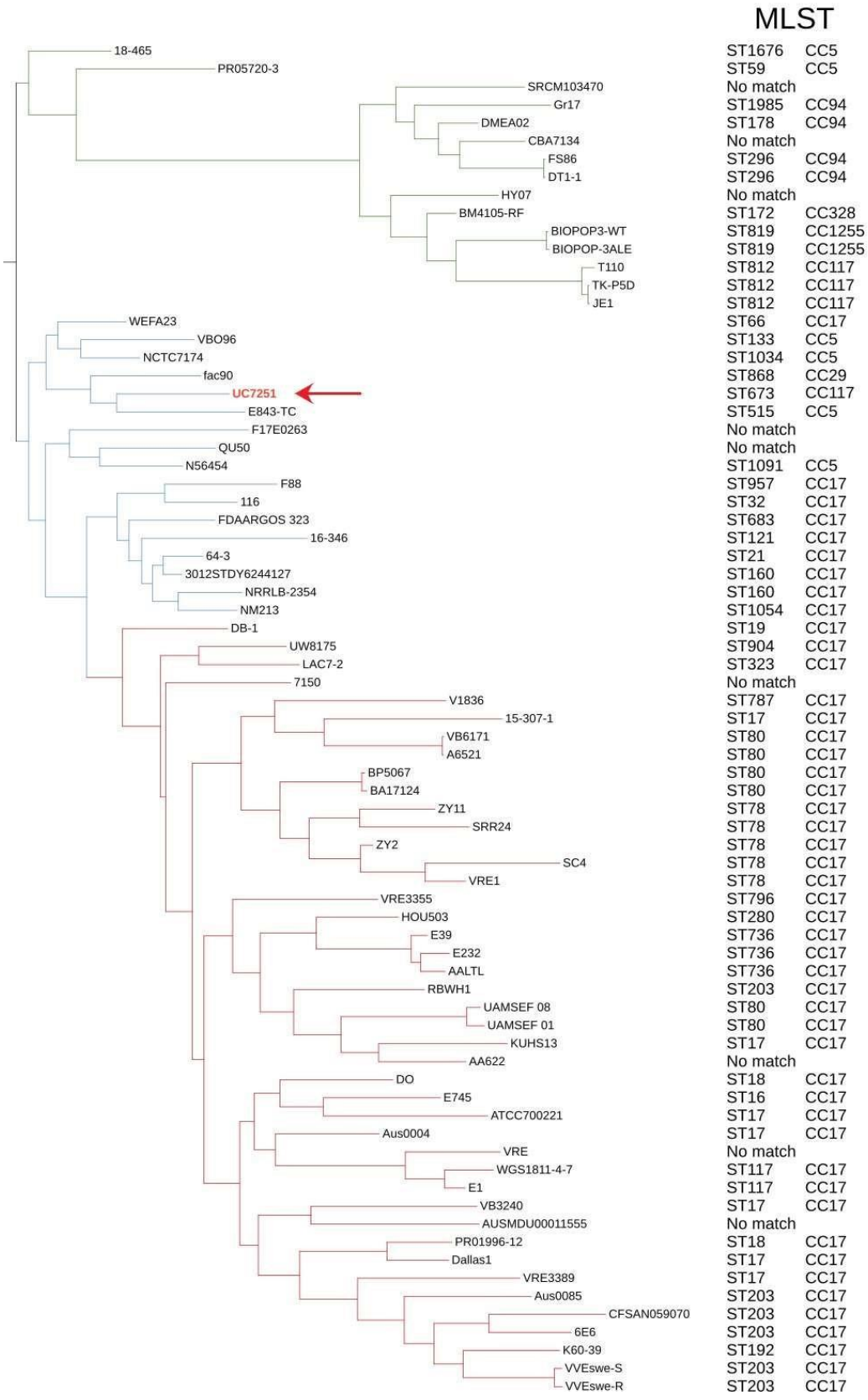
to specific environmental factors, such as antimicrobial pressure, have increased the genomic diversity through horizontal gene transfer, genome rearrangement and gene loss (63). Pan and core genome analysis uncovered an open pangenome, with a coregenome consisting of 9,5% and an accessory-genome of 90,5%. In this context, UC7251 contains 33 unique genes, mainly insertion sequences and hypothetical proteins located on the chromosome and on pUC7251_1. Transposases belonging to IS3, IS30 and IS256 families were detected as unique on both pUC7251_1 and chromosome. On the chromosome we found unique gene arnB, that catalyzes the conversion of UDP-4-ketoarabinose to UDP-4-amino-4-deoxy-L-arabinose. The modified arabinose is attached to lipid A and is required for resistance to polymyxin in Gram negative bacteria (64). Moreover, unique genes epsM and epsL coding for putative acetyltransferase and sugar transferase respectively, were detected. They are involved in the production of the exopolysaccharide (EPS) component of the extracellular matrix during biofilm formation (65). Gene cbh_2, chololyglycine hydrolyse, catalyzes the de-conjugation of bile acids (66). In *Enterococcus*, bile salt hydrolase activity has a hypo-cholesterolemic effects on animal and human hosts, conferring probiotic properties (67).

Furthermore, all 75 genomes were subjected to dDDH and ANI for genomic distance calculations. Although dDDH and ANI have different computational methods and species threshold values (70% for dDDH and 96% for ANI), they showed consistent results, confirming the taxonomical identification of UC7251. Digital DDH showed that values among UC7251-Clade A1 strains varied from 82-91%, among UC7251-Clade A2 strains 87-100% and, among UC7251-Clade B/E. lactis strains 64-70%. Similarly, ANI computation showed that the UC7251 is closest to Clade A2 strains with values between 98-100%, whereas comparison with genomes from the remaining two clades was lower (UC7251- Clade A1: 98% and UC7251-Clade B/E. lactis: 94%) (Table 2S).

The population structure and location of UC7251 was also evaluated using MLST. The genome was submitted to PubMLST and it was assigned to ST673. The latter clusters together with clonal group of ST117, which is known to be a part of CC17 meroclone (fig.1) . Published data on PubMLST showed a unique isolate harboring ST673, that contains a strain from a non-hospitalized person collected in Spain in 2010. MLST global scheme shows that UC7251, as other isolates from animal origin, belonged to hospital associated clades (68). Thus, *E. faecium* from CC17 have been also previously recovered from swine, poultry and cow samples (69–71). The use of cgMLST, a clustering based on 1423 target genes of the core genome, indicated that UC7251 belonged to the unique cluster type CT745.

Subspeciation of *E. faecium* has been also studied considering the defense mechanisms against HGT, such as CRISPR-Cas systems and R-M systems (72). CRISPR-Cas systems constitute endogenous barriers to HGT and, as a consequence, the presence of increased MGEs is associated with the complete absence or partial sequences of CRISPR-Cas systems (73). This has been observed in UC7251, where no complete CRISPR-Cas systems were detected. Differently, UC7251 carries a type I R-M system with the

allelic variations typical of clade A1 isolates, polymorphisms that are used for clade classification of *E. faecium* (74).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NCTC7174 ST1034 CC5
fac90 ST868 CC29
UC7251 ST673 CC117
E843-TC ST515 CC5
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QU50 No match
N56454 ST1091 CC5
F88 ST957 CC17
116 ST32 CC17
FDAARGOS 323 ST683 CC17
16-346 ST121 CC17
64-3 ST21 CC17
3012STDY6244127 ST160 CC17
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DB-1 ST19 CC17
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KUHS13 ST17 CC17
AA622 No match
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E745 ST16 CC17
ATCC700221 ST17 CC17
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6E6 ST203 CC17
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VVEswe-S ST203 CC17
VVEswe-R ST203 CC17

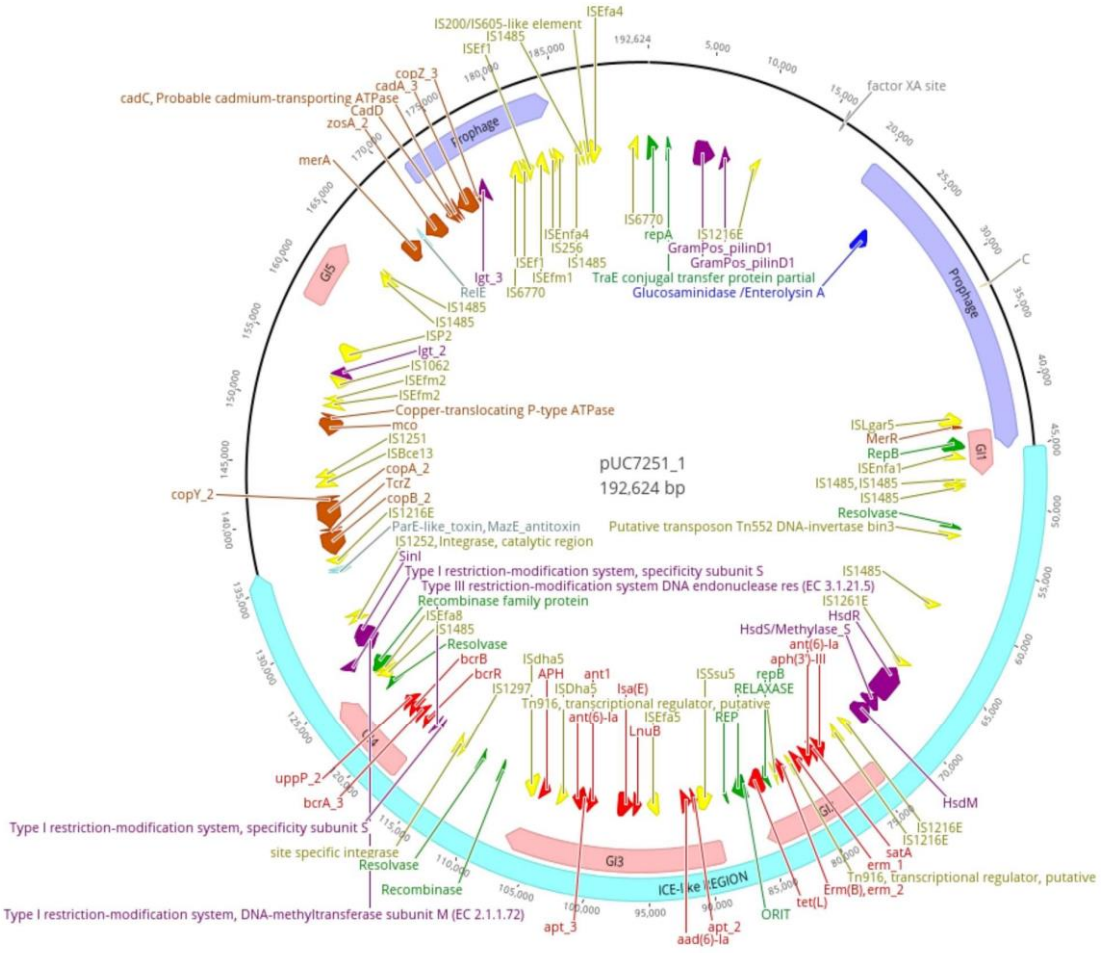
Table 2*. Distribution of virulence factors and AMR genes including antibiotic and heavy metal resistance genes in UC7251.*

Molecule	Mechanism	Gene	Locus tag or position	Product
Chromosome	Antibiotic	*AAC(6')-Ia*	UC7251_02097	Aminoglycoside N(6')-acetyltransferase (EC 2.3.1.82)
		EfmM	UC7251_02049	rRNA) methyltransferase
		liaFSR	UC7251_01795-UC7251_01797	DAP
		pbp5	UC7251_01265	penicillin binding protein 5
		tet(M)	UC7251_02367	tetracycline resistance
	Heavy Metals	cadA_1 cadA_2	UC7251_00274 UC7251_00904	Cadmium-transporting ATPase Cadmium, zinc and cobalt-transporting ATPase
		copA_1	UC7251_00909	putative copper-importing P-type ATPase A
		copB_1	UC7251_00910	Copper-exporting P-type ATPase B
		copY_1	UC7251_00907	Transcriptional repressor CopY
		copZ_1	UC7251_00275	Copper chaperone CopZ
		copZ_2	UC7251_00908	Copper chaperone CopZ
		cutC	UC7251_02237	Copper homeostasis protein CutC
		czcD	UC7251_01786	Cadmium, cobalt and zinc/H( )-K( ) antiporter
		fief	UC7251_01380	Ferrous-iron efflux pump FieF
		ftsH	UC7251_02411	Cell division-associated, ATP-dependent zinc metalloprotease FtsH
		ziaA	UC7251_01739	Zinc-transporting ATPase
		znuA	UC7251_02450	High-affinity zinc uptake system binding-protein ZnuA
		znuB	UC7251_02448	High-affinity zinc uptake system membrane protein ZnuB
		znuC	UC7251_02449	High-affinity zinc uptake system ATP-binding protein ZnuC
		zosA	UC7251_01471	Zinc-transporting ATPase
		zupT	UC7251_00019	Zinc transporter ZupT

		zur	UC7251_00846	Zinc-specific metallo-regulatory protein
	Virulence	swpB	UC7251_00118	small WxL protein B
		swpC	UC7251_00593	small WxL protein C
		swpA	UC7251_00718	small WxL protein A
		acm	UC7251_02106	cell-wall-anchored collagen adhesin, MSCRAMM
		sagA	UC7251_02425	secreted antigen A
		scm	UC7251_02536	second collagen adhesin, MSCRAMM
		efaA	UC7251_00462	adhesion associated protein
		BopD	UC7251_00373	maltose operon transcriptional repressor
		cpsA/uppS	UC7251_01047	Undecaprenyl diphosphate synthase uppS
		cpsB/cdsA	UC7251_01048	Phosphatidate cytidylyltransferase cdsA
		fms3	UC7251_00358	Efm surface protein 3 orf371 (PGC-4)
		fms12	UC7251_00496	Efm surface protein 12 orf1996 (PGC-4)
		ebpA	UC7251_00550	PGC-3: endocarditis- and bio- film-associated pili A (MSCRAMM)
		epbB	UC7251_00551	PGC-3: endocarditis- and bio- film-associated pili B (MSCRAMM)
		ebpC	UC7251_00552	PGC-3: endocarditis- and bio- film-associated pili C (MSCRAMM)
		srtC	UC7251_00553	sortase C
		fms6	UC7251_00720	Efm surface protein 6 LPXTG family cell surface proteinPGC-4)
		fms7	UC7251_01220	Efm surface protein 7 orf2356 (PGC-4)
		fms22	UC7251_01278	Efm surface protein 22 orf884 (PGC-4)
		yidC	UC7251_00884	inner memebrane protein translocase and chaperone
pUC7251_1	Antibiotic	ant(6)-Ia	UC7251_02669	Aminoglycoside 6-adenylyltransferase
		ant1	UC7251_02694	Streptomycin 3"-adenylyltransferase
		ant(6)-Ia	UC7251_02696	Aminoglycoside 6-nucleotidyltransferase

		aph	UC7251_02698	aminoglycoside phosphotransferase family protein
		Lnu(B)	UC7251_02689	lincosamide nucleotidyltransferase
		lsa(E)	UC7251_02690	ABC-F type ribosomal protection protein Lsa(E)
		tet(L)	UC7251_02678	tetracycline efflux MFS transporter Tet(L)
		satA	UC7251_02668	Streptothricin acetyltransferase A
		erm_1	UC7251_02671	rRNA adenine N-6-methyltransferase
		erm_2	UC7251_02674	rRNA adenine N-6-methyltransferase
		aad(6)-Ia	UC7251_02684	Aminoglycoside 6-adenylyltransferase
	Heavy Metals	copZ_3 cadA	UC7251_02781 UC7251_02780	Copper chaperone CopZ Cadmium, zinc and cobalt-transporting ATPase
		cadC	UC7251_02779	Cadmium, zinc and cobalt-transporting ATPase
		cadD	UC7251_02778	Cadmium, zinc and cobalt-transporting ATPase
		copA_2	UC7251_02740	Copper-exporting P-type ATPase
		copB_2	UC7251_02739	Copper-exporting P-type ATPase B
		copY_2	UC7251_02742	Transcriptional repressor CopY
		mco	UC7251_02750	Multicopper oxidase mco
		merA	UC7251_02772	mercuric reductase
		merR1	UC7251_02771	Mercuric resistance operon regulatory protein
		TcrZ	UC7251_02740	copper chaperone
		zosA	UC7251_02776	Zinc-transporting ATPase
	Virulence	lgt	UC7251_02756, UC7251_02782	surface protein anchor
		fms20	UC7251_02583- UC7251_02588	PGC-1: surface protein 20
		fms21 or pilA	UC7251_02583- UC7251_02588	PGC-1: surface protein 21

A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ISEfa4
IS200/IS605-like element
IS1485
ISEf1
copZ_3
cadA_3
cadC, Probable cadmium-transporting ATPase
CadD
zosA_2
merA
Prophage
factor XA site
IS6770
repA
IS1216E
GramPos_pilinD1
GramPos_pilinD1
ISEnfa4
IS256
ISEf1
IS1485
ISEfm1
TraE conjugal transfer protein partial
Glucosaminidase /Enterolysin A
Prophage
GI5
Igt_3
RelE
IS6770
IS1485
IS1485
ISP2
Igt_2
IS1062
ISEfm2
ISEfm2
Copper-translocating P-type ATPase
mco
IS1251
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copA_2
TcrZ
copB_2
IS1216E
ParE-like_toxin, MazE_antitoxin
IS1252, Integrase, catalytic region
copY_2
SinI
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Type III restriction-modification system DNA endonuclease res (EC 3.1.21.5)
Recombinase family protein
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IS1485
Resolvase
bcrB
bcrR
ISLgar5
MerR
RepB
ISEnfa1
IS1485, IS1485
IS1485
Resolvase
Putative transposon Tn552 DNA-invertase bin3
GI1
IS1485
IS1261E
HsdR
HsdS/Methylase_S
ant(6)-Ia
aph(3')-III
ISDha5
APH
ant1
IS1297
ISDha5
Isa(E)
Tn916, transcriptional regulator, putative
ant(6)-Ia
ISSsu5
repB
RELAXASE
REP
ISEfa5
LnuB
uppP_2
bcrA_3
Type I restriction-modification system, specificity subunit S
site specific integrase
Resolvase
Recombinase
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GI4
GI3
ICE-like REGION
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apt_2
aad(6)-Ia
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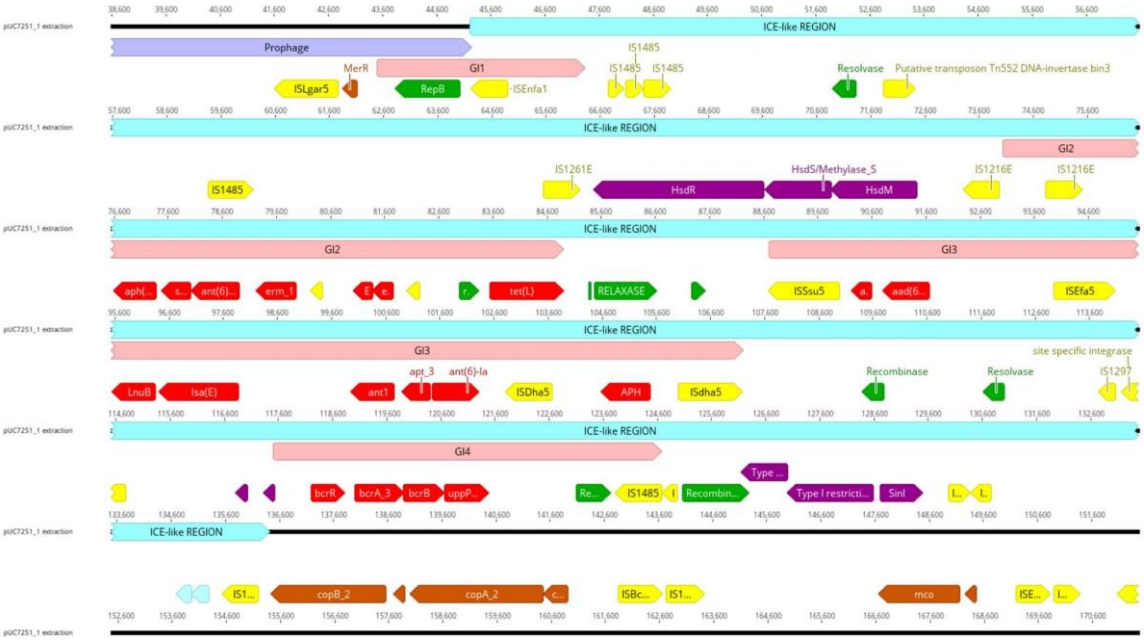
erm_1
satA
IS1216E
IS1216E
HsdM
Tn916, transcriptional regulator, putative

B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Prophage
GI1
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RepB
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Resolvase
Putative transposon Tn552 DNA-invertase bin3
GI2
GI3
HsdR
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RELAXASE
ISSsu5
ISEfa5
GI4
site specific integrase
Recombinase
Resolvase
LnuB
Isa(E)
ISDha5
APH
apt_3
ant(6)-Ia
tet(L)
erm_1
aph(...
ant(6)...
aad(6...
IS1297
bcrR
bcrA_3
bcrB
uppP...
IS1485
Recombin...
Type I restricti...
SinI
copB_2
copA_2
mco
ISE...

***Figure 2**. A. Map of plasmid pUC7251_1 harboring one large containing prophage sequences (mauve), an integrative conjugative-like element (cyan), five genomic islands (pink), insertion sequences (yellow), antibiotic resistance genes (red), metal resistance genes (orange), virulence factors (dark violet) and replication initiation systems (green), toxin-antitoxin systems (light blue). B. Details of the ICE-like region. The genetic elements are indicated with the color code above mentioned.*

4.4. Conjugation experiments suggest AMR gene transfer by Insertion Sequences

The MDR profile of UC7251 endorsed the further evaluation of transmissibility of AMR genes and we focused on tetracycline resistance coded by two genes on Tn*916* and pUC7251_1 and the plasmid encoded erythromycin resistance. This was tested through conjugation experiments where gene exchange was demonstrated at inter- and intra-generic level (Table 3 and Table 3S). Filter mating experiments demonstrated that tetracycline resistance was transferred from UC7251 to *E. faecalis* OG1rf, L. innocua L7, L. monocytogenes DSM 15675, S. aureus UC7180, L. rhamnosus UC8647, with frequencies of transconjugants per donors varying from 6 x 10-3 to 5,7 x 10-6 CFU/ml. No gene transfer was observed toward Gram negative species. The transfer of the tetM gene was confirmed by PCR assays, whereas tetL was absent in all tetracycline positive transconjugants. The transfer of the tetM gene was found to be carried by chromosomal transposon Tn*916* from *E. faecalis*. This operon was predicted in chromosomal locus UC7251_02362-02376. The nucleotide identity between the 18,032 bp sequence of Tn*916* of UC7251 and *E. faecalis* (Genbank Accession No. U09422.1) sequences, was of 99.97%. It has been discovered that the presence of subinhibitory concentrations of specific classes of antibiotics can trigger the mobility of Tn*916*, as it has a broad inducibility of antibiotic resistance genes, implying that the dissemination of resistance genes is not necessarily linked to their selective pressure (103).

No gene transfer for the genes coding for erythromycin resistance was observed, consistently with the characteristics of pUC7251_1, a mobilizable but non conjugative plasmid lacking the complete conjugation apparatus.

***Table 3**. Conjugation of tetracycline resistance between E. faecium UC7251 and strains from other genera.*

Donor	Recipient Strain	Conjugation Frequency (T/D)	PCR Confirmation	
			*tet*M	*tet*L
E. faecium UC7251	*E.s faecalis* OG1rf	6.01E-03 5.68E-06	+	-
	L. innocua L7		+	-
	L. monocytogenes DSM 15675	8.38E-04	+	-
	S. aureus UC7180	3.78E-02	+	-
	L. rhamnosus UC8647	6.84E-05	+	-

4.5. **Virulence markers in the UC7251 genome show a collection of colonization facilitators**

The complete assembly and annotation of UC7251 genome allowed investigation of the presence of putative virulence markers (Table 2). Adherence is an essential step in bacterial pathogenesis, required for colonization and attachment and it is therefore considered a type of virulence marker. When scrutinizing the genome of UC7251, several microbial surface components, recognizing adhesive matrix molecules (MSCRAMMs) including LPXTG family cell wall-anchored surface proteins as well as fimbriae proteins such as pili, were identified. It is important to denote the presence of genes acm (cell-wall-anchored collagen adhesin) and scm (second collagen adhesin). These proteins enhance initial adherence in vivo and interact with extracellular matrix components. Other genes associated with adhesion, efaA (*E. faecium* surface protein) and sagA (secreted antigen A) were detected. A novel class of cell surface proteins coded by WxL operon, found in clade A *E. faecium* isolates, with a functional role in virulence associated with endocarditis pathogenesis and bile salt resistance was previously investigated (104). The coding genes swpA (small WxL protein A), swpB (small WxL protein B) and swpC (small WxL protein C) were found in UC7251. Additionally, malR, a maltosebinding transcriptional regulator that increases biofilm production in the presence of this specific carbohydrate, was detected. Pili associated proteins, previously described as Pilin Gene Clusters (PGC-1, PGC-2, PGC-3, PGC-4), (16),were identified. PGC-1 is composed by the genes fms20 and fms21; both are present along with a sortase A. This loci/operon is located between UC7251_02853 and UC7251_02588 in pUC7251_1. In addition, PGC-3 was found with 100% of nucleotide identity containing the endocarditis and biofilm associated pili genes ebpA, ebpB, ebpC accompanied by srtC (sortase) and flanked by IS1216E. This region is encompassed from UC7251_02583 to UC7251_02589 in the chromosome. PGC-4 cluster is incomplete lacking operon fms11-19-16 and PGC-2 associated genes fms14-17-13. UC7251 does not express the capsular polysaccharide, presenting the capsule operon polymorphism CPS type 1 (105) and does not harbor cytolysin (106) and BoNT/En toxin, a botulin type toxin found in a single strain of *E. faecium* (107). *E. faecium* UC7251 lacks the putative HA virulence markers as defined by EFSA (4) and does not harbor the complete operons coding pili-associated proteins, which is typical of clade A1 isolates

5. CONCLUSION

The presence of multi-drug resistant strains in ready-to-eat fermented food represents a risk of public health for the spread of AMR determinants in the food chain and in the gut microbiota of consumers. In silico bioinformatic evaluations derived from genomic data permitted to accurately assess the safety of UC7251, a strain of *E. faecium* clade A2 which does not carry virulence factors typical of HA strains but presents the colocation of several antimicrobial resistance genes with heavy metal resistances on the mobilizable plasmid pUC7251_1 and the conjugative transposon Tn*916*.

This work emphasizes the importance of a surveillance for the presence of AMR bacteria in food, with particular attention to fermented RTE foods. Moreover, the presence of MDR strains carrying mobile AMR genetic elements incites the development of innovative strategies for the mitigation of the risk related to antimicrobial resistance diffusion in food.

6. TRANSPARENCY DECLARATION

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7. AUTHOR CONTRIBUTIONS

MB: methodology, formal analysis, investigation, resources, and writing—original draft preparation. GM: methodology, investigation, and writing—original draft preparation. EP: methodology, investigation, resources, and writing—original draft preparation. CC: writing—review and editing. DB: writing—review and editing, visualization, and supervision. PC: conceptualization, writing—review and editing, validation, visualization, supervision, and project administration. All authors contributed to the article and approved the submitted version.

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9. SUPPLEMENTARY MATERIALS

***Table 1S**. Primers used in this work*

Scope	Primer name	Primer sequence (5'-3')	Reference
Species identification	EM1A (ddl gene)	TTGAGGCAGACCAGATTGACG	Cheng et al. 1997
	EM1B (ddl gene)	TATGACAGCGACTCCGATTC	
Screening of HA markers	esp14F	AGATTTCATCTTTGATTCTTGG	Leavis et al., 2003
	esp12R	AATTGATTCTTTAGCATCTGG	
	hylEfm-F	GAGTAGAGGAATATCTTAGC	Rice et al., 2003
	hylEfm-R	AGGCTCCAATTCTGT	
	IS16-F	CATGTTCCACGAACCAGAG	Werner et al., 2011
	IS16-R	TCAAAAAGTGGGCTTGGC	
AMR genes	Efmpbp5-1outsideF	GGAATGACAAGCAAGAGAAGGAGG	Galloway-Pena et al., 2011
	Efmpbp5-1F	ATGAAAAGAAGTGACAAGCACGGC	
	Efmpbp5-1R	GCAAAGATGAATACCTCATTAGG	
	Efmpbp5-2F	CAAAGTAATCGGGTTGTACCCAGC	
	Efmpbp5-2R	GTCCCACGAAGATCCTTATCAAAAGCC	
	Efmpbp5-3F	GGCTTTTGATAAGGATCTTCGTGGGAC	
	Efmpbp5-3R	CCCATTTTCAACGTTTCTTGTGCC	
	Efmpbp5-4F	GGCACAAGAAACGTTGAAAATGGG	
	Efmpbp5-4R	TTATTGATAATTTTGGTTGAGGTATTG	
	Efmpbp5-4outsideR	CGCCACAGTCCTTTTACTGTAC	
	Rpbp5_1F	GCAAAGATGAATACCTCATTAGG	
	Rpbp5_1R	CAAAGTAATCGGGTTGTACCCAGC	
	Rpbp5_2F	CAGAACTTCCAGCTGGAGCTAC	
	Rpbp5_2R	GATCATAGCTTGGAGAGCTAGC	
	Rpbp5_3F	GCGACAGGTTATGCTCCTGG	
	Rpbp5_3R	GAATACATTGCTGCTTGCTGGATAGG	

	ermB1	GAAAAGGTACTCAACCAAATA	Sutcliffe et al., 1996
	ermB2	AGTAACGGTACTTAAATTGTTTAC	
	tetM1	GAACTCGAACAAGAGGAAAGC	Olsvik et al., 1995
	tetM2	ATGGAAGCCCAGAAAGGAT	
	tetL-up	ATAAATTGTTTCGGGTCGGTAAT	Trzcinski et al., 2000
	tetL-rev	AACCAGCCAACTAATGACAATGAT	
	aad6_F	TTCGAATTGTGACCCTTGAG	This study
	aad6_R	TGGTTCAGATGATCGATTGC	
	aph3-IIIa_F	GCCGATGTGGATTGCGAAAA	Ouoba et al., 2008
	aph3-IIIa_R	GCTTGATCCCCAGTAAGTCA	
	aadE_F	ATGGAATTATTCCCACCTGA	Ouoba et al., 2008
	aadE_R	TCAAAACCCCTATTAAAGCC	
	SatA_F	TCAAAGTTGGCGTATAA	Jacob et al., 1994
	SatA_R	TAAACCCAGCGAACCAT	
	Ant(6)-Ia_F	GCCTTTCCGCCACCTCACCG	Swenson et al., 1995
	Ant(6)-Ia_R	ACTGGCTTAATCAATTTGGG	
	Lnu-B_F	ATCGAGCAGTGGTCTTTGCA	This study
	Lnu-B_R	GGTTGTTTGACGTAGCTCCG	
	IsaE_F	TTGGCACGTTTCATCGCTTT	This study
	IsaE_R	ACGGACGCGGTAAAACTACT	

Table 2S*. selected strains for taxonomic and phylogenetic analyses*

Strain	Clade	Genbank Accesion	MLST	Clonal Complex according to MLST scheme and central ST	dDDH Average against UC7251	ANI against UC7251
7150	A1	GCA_019356355.1	No match		90.53	98.93
15-307-1	A1	GCA_002973755.2	17	CC17	82.73	98.57
6E6	A1	GCA_001518735.1	203	CC203	82.40	98.58
A6521	A1	GCA_012933195.2	80	CC80	84.63	98.60
AA622	A1	GCA_019977575.1	No match		86.83	98.78

AALTL	A1	GCA_002880635.1	736	CC17	87.27	98.58
ATCC700221	A1	GCA_001594345.1	17	CC17	86.80	98.89
Aus0004	A1	GCA_000250945.1	17	CC17	86.03	98.78
Aus0085	A1	GCA_000444405.1	203	CC203	82.27	98.67
AUSMDU00011555	A1	GCA_017301355.1	No match		82.73	98.54
BA17124	A1	GCA_012932975.2	80	CC80	85.50	99.04
BP5067	A1	GCA_012932985.2	80	CC80	85.60	99.00
CFSAN059070	A1	GCA_003071425.1	203	CC203	82.63	98.59
Dallas1	A1	GCA_015999605.1	17	CC17	83.73	98.51
DB-1	A1	GCA_006337045.1	19	CC64	87.20	98.92
DO	A1	GCA_000174395.2	18	CC18	87.33	98.80
E1	A1	GCA_001886635.1	117	CC117	83.27	98.62
E232	A1	GCA_002777275.1	736	CC17	86.43	98.58
E39	A1	GCA_001635875.1	736	CC17	86.50	98.60
E745	A1	GCA_001750885.1	16	CC17	84.17	98.84
HOU503	A1	GCA_005952885.1	280	CC280	87.90	98.65
K60-39	A1	GCA_002334625.1	192	CC192	85.60	98.71
KUHS13	A1	GCA_009938285.1	17	CC17	87.00	98.77
LAC7-2	A1	GCA_009036045.1	323	CC17	89.57	98.87
PR01996-12	A1	GCA_018219325.1	18	CC18	84.57	98.66
RBWH1	A1	GCA_003957785.1	203	CC203	85.17	98.66
SC4	A1	GCA_002848385.1	78	CC78	84.90	98.72
SRR24	A1	GCA_009734005.2	78	CC78	85.23	98.77
UAMSEF_01	A1	GCA_005886545.1	80	CC80	87.50	98.70
UAMSEF_08	A1	GCA_005886655.1	80	CC80	87.50	98.67
UW8175	A1	GCA_001587115.1	904	CC280	91.23	98.91
V1836	A1	GCA_008728455.1	787	CC80	85.43	98.75
VB3240	A1	GCA_005576735.1	17	CC17	85.73	98.55
VB6171	A1	GCA_017897965.1	80	CC80	84.63	98.60

VRE	A1	GCA_009697285.1	No match		82.70	98.69
VRE1	A1	GCA_006007925.1	78	CC78	83.27	98.63
VRE3355	A1	GCA_017584065.1	796	CC203	86.43	98.84
VRE3389	A1	GCA_015999405.1	17	CC17	85.43	98.71
VVEswe-R	A1	GCA_007917035.3	203	CC203	82.70	98.56
VVEswe-S	A1	GCA_007917315.3	203	CC203	82.87	98.58
WGS1811-4-7	A1	GCA_016864255.1	117	CC117	84.50	98.69
ZY11	A1	GCA_009938075.1	78	CC78	88.07	98.80
ZY2	A1	GCA_010120755.1	78	CC78	84.93	98.81
116	A2	GCA_018279145.1	32	CC32	88.03	98.90
16-346	A2	GCA_002761555.1	121	CC78	87.60	99.07
18-465	A2	GCA_018516845.1	1676	CC5	89.77	99.10
3012STDY6244127	A2	GCA_900683475.1	160	CC32	91.10	99.16
64/3	A2	GCA_001298485.1	21	CC32	89.43	98.99
E843-TC	A2	GCA_019774555.1	515	CC5	90.57	99.18
F17E0263	A2	GCA_006280355.1	No match		87.27	98.65
F88	A2	GCA_019175425.1	957	CC323/CC17	89.57	98.85
fac90	A2	GCA_016743855.1	868	CC29	92.47	98.91
FDAARGOS_323	A2	GCA_002983785.1	683	CC32	90.00	98.80
N56454	A2	GCA_006351845.1	1091	CC5	87.60	98.70
NCTC7174	A2	GCA_900637035.1	1034	CC5	91.87	99.21
NM213	A2	GCA_005166365.1	1054	CC32	89.10	98.90
NRRLB-2354	A2	GCA_001544255.1	160	CC32	89.60	99.05
QU 50	A2	GCA_006741355.1	No match		87.97	98.62
UC7251	A2	GCA_000411655.2	673	CC117	100.00	100.00
VBO96	A2	GCA_019456555.1	133	CC5	92.10	99.08
WEFA23	A2	GCA_002850515.1	66	CC66	91.87	99.11
BIOPOP-3 ALE	B	GCA_012045365.1	819	CC1255	69.07	94.37
BIOPOP3-WT	B	GCA_012045505.1	819	CC1255	69.07	94.34
BM4105-RF	B	GCA_003269465.1	172	CC328	64.63	94.25
CBA7134	B	GCA_004015145.1	No match		70.13	94.80
DMEA02	B	GCA_008330605.1	178	CC94	69.47	94.80

DT1-1	B	GCA_011745645.1	296	CC94	68.40	94.49
FS86	B	GCA_013201055.1	296	CC94	68.40	94.54
Gr17	B	GCA_003711605.1	1985	CC94	68.17	94.60
HY07	B	GCA_003574925.1	No match		68.47	94.48
JE1	B	GCA_003667965.1	812	CC117	67.70	94.66
SRCM103470	B	GCA_004103475.1	No match		69.30	94.83
T110	B	GCA_000737555.1	812	CC117	66.50	94.70
TK-P5D	B	GCA_015377765.1	812	CC117	67.70	94.65
PR05720-3	B	GCA_018219285.1	59	CC5	82.50	98.15

Table 3S*. Strains used for mating experiments with tetracycline and erythromycin.*

Conjugation			Tetracycline				Erythromycin		
			Conjugation frequency		PCR confirmation		Conjugation frequency		PCR confirmation
Donor	Recepient	Strain	(T/D)	(T/R)	tetM	tetL	(T/D)	(T/R)	ermB
E. faecium UC7251	*Enterococcus faecalis*	OG1rf	6.01E03	2.80E-06	yes	no	/	/	no
	Listeria innocua	L7	5.68E06	7.16E-05	yes	no	/	/	no
	Listeria monocytogenes	DSM 15675	8.38E-04	3.88E-03	yes	no	/	/	no
	Staphylococcus aureus	UC7180	3.78E02	1.37E-01	yes	no	/	/	no
	Staphylococcus xylosus	UC8727	/	/	/	/	/	/	/
	Staphylococcus carnosus	UC8838	/	/	/	/	/	/	/
	Lactobacillus rhamnosus	UC8647	6.84E05	1.30E-04	yes	no	/	/	/
	Lactobacillus paracasei	UC8477	/	/	/	/	/	/	/
	Lactobacillus casei	UC8477	/	/	/	/	/	/	/
	Lactobacillus fermentum	UC10045	/	/	/	/	/	/	/
	Lactobacillus plantarum	UC8479	/	/	/	/	/	/	/
	Lactobacillus reuteri	UC10043	/	/	/	/	/	/	/

Bacillus cereus	UC4044	/	/	/	/	/	/	/
Escherichia coli	BL21	/	/	/	/	/	/	/
Pseudomonas koreensis	Psk	/	/	/	/	/	/	/
Pseudomonas aeruginosa	Psa	/	/	/	/	/	/	/
Pseudomonas chlororaphis	Psc	/	/	/	/	/	/	/
Pseudomonas putida	Psp	/	/	/	/	/	/	/
Pseudomonas fluorescens	Psf	/	/	/	/	/	/	/
Weisella confusa	LMG 18478	/	/	/	/	/	/	/
Weisella confusa	LMG17696	/	/	/	/	/	/	/
Weisella confusa	LMG 17695	/	/	/	/	/	/	/
Weisella confusa	BCC 2344	/	/	/	/	/	/	/
Weisella confusa	BCC 3263	/	/	/	/	/	/	/
Weisella confusa	BCC4255	/	/	/	/	/	/	/
Weisella confusa	024F6	/	/	/	/	/	/	/
Pediococcus pentosaceus	UC8487	/	/	/	/	/	/	/
Pediococcus acidilactici	UC8715	/	/	/	/	/	/	/
Clostridium tyrobutyricum	UC7086	/	/	/	/	/	/	/
Clostridium sporogenes	UC9000	/	/	/	/	/	/	/

T*able 4S*. *Annotation of ICE and IME in UC7251*

	Chromosome		pUC7251_1
	Region 1	Region 2	Region 1
Location (nt)	1097029-1202552	2360857-2421852	45229..136214
Length (bp)	105524	60996	90986
GC content (%)	39.28	38.52	35.40
oriT	-	2419860-2419992	85370..85407

Insertion site	Aspartate racemase (1096652-1097383)		hypothetical protein (2421842-2422156)		-	
Direct Repeats	attL: 1097029-1097044 (aacagaaggaagtatg)/attR: 1202537-1202552 (aacagaaggaagtatg)		attL: 2360857-2360872 (tttctttattctttta)/attR: 2421837-2421852 (tttctttattctttta)		-	
Type	Putative IME		Putative ICE with T4SS		ICE-like region	
Annotations	Proteins	Position	Proteins	Position	Proteins	Position
	T2SSE	1119712-1120962	T2SSE	2384650-2386434	Integrase	4522-45909
	FtsK_SpoIIIE (T4CP)	1121045-1121632	TrbC	2393945-2394601	Pfam-B_706	47135-47335
	Pfam-B_6973 (Relaxase)	1123703-1124461	T2SSE	2396328-2398097	Integrase	48425-48928
	T2SSE	1140006-1141790	Integrase	2404634-2405851	Integrase	59358-60194
	VirB3	1181314-1181997	tet(M)	2408450-2410369	Integrase	61549-62634
			TrbL (Orf15_Tn, T4SS component)	2412673-2414850	Integrase	64313-64867
			AAA_10 (Orf16_Tn, T4SS component)	2414853-2417300	Integrase	65555-66235
			TrbC (Orf19_Tn, T4SS compont)	2418379-2418600	Integrase	73299-73979
			Rep_trans (Relaxase)	2418643-2419848	Integrase	74831-75511
			FtsK_SpoIIIE (T4CP)	2420026-2421411	Mob_Pre (Relaxase)	85476-86624
					Recombinase (Integrase)	107823-109391
					Recombinase (Integrase)	109393-109809
					Integrase	113755-114075
					Integrase	114194-114784
					Integrase	123820-124686
					Recombinase (Integrase)	125063-126289
					Integrase	135534-136214

CHAPTER 3

Wild boars as an indicator of environmental spread of ESβLs-producing *Escherichia coli.*

Wild boars as an indicator of environmental spread of ESβLs-producing *Escherichia coli*

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1. ABSTRACT

The Antimicrobial Resistance (AMR) represents an increasing issue worldwide, spread not only in humans and farmed animals, but also in the wildlife. One of the most relevant problems is represented by Extended Spectrum Beta-Lactamases (ESβLs) producing *Escherichia coli*, because they are cause of important infections in humans. Wild boars (*Sus scrofa*) as a source of ESβLs attracted attention because of the increasing density and their habits that lead them to be at the human-livestock-wildlife interface. The aim of this study is to increase the knowledge about the ESβLs *E. coli* strains carried by wild boars living in a particularly high-density area of Northern Italy. The analysis of 60 animals allowed to isolate 16 ESβLs-producing *E. coli* strains (prevalence 23.3%), which were characterized from a phenotypical and molecular point of view. The overall analysis revealed that the 16 isolates were all not only ESβLs producers, but also multi-drug resistant and carried different types of plasmid replicons. The genome analysis performed on a subset of isolates confirmed the heterogeneity observed with PFGE and highlighted the presence of two pandemic sequence types, ST131 and ST10, with different collection of virulence factors. The genomic context of ESβLs genes further evidenced that all of them were surrounded by transposons and insertion sequences, suggesting the possibility to exchange AMR genes.

Overall, this study showed the worrying dissemination of ESβLs-producing *E. coli* in wild boars in Northern Italy, suggesting the role of these animals as spreader of AM resistance and their inclusion in surveillance programs, to shed light to the "One Health" complex interactions.

2. INTRODUCTION

The concept of One Health is based on an existing connection between humans, animals, plants, and environmental health (1). In recent years, this concept has been reformulated underlying the role of interconnected ecosystems, i.e. geographically close systems, in the occurrence of common health traits (2). Such interactions between the different domains are supported by the exchange of microbial communities between humans, animals, plants, and the local environment, thus influencing the health conditions of organisms, communities, and ecosystems (2). In this context, despite the importance of microbial communities' distribution across domains, AMR is considered the quintessential One Health issue (3).

The emergence and distribution of antimicrobial-resistant bacteria (AMB) between people and animals has been often attributed to abuse/misuse of antimicrobials in husbandry practices (sub-therapeutical doses and long-lasting treatments), which create the ideal conditions for bacteria to develop resistance (3). AMB shed by farmed animals may contaminate agricultural areas through spreading of manure and reach surface water, thus contributing to contamination of soil, plants, and wild animals (4). On the other hand, important sources of AMR bacteria are represented by hospitals, which release their sewage drains and wastewaters in the environment and represent a menace for different ecosystems (5). Even if hospital

effluents are treated in wastewater treatment plants (WWTPs), AMR bacteria and their resistant genes can survive (6), thus persisting in the environment and circulating in the ecosystems.

It is commonly assumed that this anthropogenically-derived pollution of antimicrobials, AMB and resistance genes from human waste and livestock farms is the source for wildlife contamination (7). As a matter of fact, wildlife commonly carries AMB deriving from contact with anthropogenic sources that pollute the environment with AMB and/or with antimicrobials (8–10). Nevertheless, despite the isolation of AMB, some studies suggest a lack of evidence of direct contact of wild animals with human or livestock sewage, slurry, or faeces, thus questioning clear pathways of AMR transmission (9).

Most of the studies in wild animals are focused on the detection of *E. coli* producing ESβLs, which are known to be emerging in livestock as well as in wildlife, thus escaping from human clinical settings (11–14). It has been reported that occurrence of ESβLs-producing *E. coli* in wild animals, especially wild birds, could be a spill-over form of environmental pollution from human and livestock sources (11). As a matter of fact, wild birds have been considered environmental indicators, reservoirs and even spreaders of AMR (15). However, the role of AMR indicators could be postulated also for wild mammals, as wild boars (*Sus scrofa*).

During the last decades, wild boars have been expanding in Europe despite they are one of the most intensively hunted ungulate species (16). Due to their high reproductivity rate and omnivorous habits, their population is likely to overgrowth in many territories (16,17). In Italy, wild boars are among the most common wild ungulates, with densities varying from 0.01–0.05 to 2.32–10.5 animals/km2 across the whole Italian territory (18). In the region of the study (Emilia-Romagna region; 22,451 km2), a density of 1.37–2.31 wild boars/km2 was estimated, thus consisting in a regional wild boar population ranging between thirty and fifty thousand animals. Since these animals are also used for production of non-thermally treated foods, such as cured meats and dry fermented sausages, it is possible for bacteria from animals to reach the consumers via these foods.

The main aims of the study were the evaluation of: (i) prevalence of ESβLs-producing *E. coli* among a small group of wild boars hunted in Northern Italy (Emilia-Romagna region); (ii) molecular typing of the isolates to characterize AMR determinants, virulence determinants and phylogenetic groups; (iii) comparison with strains isolated from different sources, including human, food, wild and companion animals.

3. MATERIALS AND METHODS

3.1. Samples collection and isolation of ESβLs-producing *E. coli* strains

From June to December 2018, 60 wild boars (35 females and 25 males) hunted in Parma province, Northern Italy, were tested for the presence of ESβLs-producing *E. coli*. Fourteen animals (23.3%)

belonged to age class 0 (young; less than 12 months), 17 (28.3%) to age class 1 (subadults; 13-24 months) and 29 (48.3%) to age class 2 (> 24 months). For this survey only animals dead since less than 5 hours were selected. Mesenteric lymph nodes (MLNs) and faecal samples were collected immediately after evisceration. MLNs were washed with sterile saline solution and decontaminated using ethyl alcohol before being placed in sterile containers. Faecal samples were collected from the colon and placed in sterile containers. The samples were transported to the laboratory at refrigerated conditions and tested the day of collection.

MLNs were cut in small pieces (0.1-0.2 cm) by using sterile scissors. Five to 10 g grams of MLNs, according to their size, and 10 g of faeces were diluted 1:10 in Buffered Peptone Water (BPW; Oxoid, Basingstoke, UK) and incubated at 37±1°C for 18-20 h. A 100 µL loopful of the cultures was streaked onto MacConkey (Oxoid) agar plates added with a disk containing cefotaxime (CTX; 5 µg) and incubated at 44±1°C for 21±3 h. Colonies grown in proximity of the antimicrobial disk were selected to be seeded onto Tryptone Soya Agar (TSA, Oxoid) and Tryptone Soya Broth (TSB, Oxoid), and incubated at 44±1°C for 21±3 h. Indole production was tested by adding James' reagent to TSB cultures. From TSA plates of indole-positive cultures, one well isolated colony was identified at species level with the Microgen® GN-A (Biogenetics, Padua, Italy) system. *E. coli* isolates were analysed by the Kirby-Bauer test following EUCAST recommendations (2018) using disks produced by Oxoid containing cefotaxime (CTX; 5 µg), ceftazidime (CAZ; 10 µg). *E. coli* ATCC 25922 was used as a quality control microorganism. The strains which showed an inhibition zone diameter <17 mm for CTX and <19 mm for CAZ were selected for ESβLs testing. Phenotypic identification of ESβLs-producing *E. coli* was performed using the ESβLs-Confirm Kit (Rosco Diagnostica, Taastrup, Denmark) following the manufacturer's instructions.

3.2. Antimicrobial susceptibility test

The antimicrobial susceptibility profiles for the phenotypically identified ESβLs-producing *E. coli* isolates were determined using Microscan Gram-negative MIC/Combo panels and analyzed through the semi-automated system MicroScan autoSCAN-4 (Beckman Coulter) following manufacturer's instructions. Clinical categorization of the isolates was performed based on the EUCAST 2019 clinical breakpoints (http://www.eucast.org).

3.3. Molecular investigation of resistance determinants

The presence of ESβLs and carbapenemases determinants was investigated by microarray Check-Points CT 103 XL Check-MDR assay (Wageningen, the Netherlands), and PCR. To determine the exact allelic variant of *bla*$_{\text{CTX-M}}$, two-directional DNA sequencing was performed. PCR amplicons of 593 bp, obtained using the primers pair Fw: 5'-ATGTGCAGYACCAGTAARGT-3 and Rev: 5'-

TGGGTRAARTARGTSACCAGA-3', were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). DNA Sequencing was performed using the Microsynth services (Macrosynth Seqlab, Germany). The alignment between the forward, reverse, and reference DNA sequences were accomplished using Chromaspro software (2.1.10) and analysed using the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). PCR assays were performed to assess the presence of resistance determinants against quinolones (*qnrB*, *qnrS* and *aac(6')-Ib-cr*), and aminoglycosides (*aadA*, *armA*, *rmtB*, and *rmtC*).

3.4. Conjugation assay and plasmid typing

To assess the possible transferability of the blaCTX-M determinants identified, conjugation assays in liquid were performed using three different *E. coli* strains as recipients.: included the *E. coli* K12 strain J62 (pro-, his-, trp-, lac-, Sm R), J53-2 (met-, pro-, rif R), and the J53 AzideR. Donor strains in logarithmic growth phase were mixed with recipients in the early stationary phase in a 1:10 ratio in Mueller Hinton broth, and the mixture was incubated at 37°C overnight. The transconjugants were selected on MacConkey agar containing cefotaxime (2 mg/L) plus streptomycin (1000 mg/L), rifampicin (100 mg/L) or sodium azide (100 mg/L) (Tartor et al. 2021). The detection sensitivity of the assay was approximately 10−8 transconjugants per recipient. At least three possible transconjugants colonies for each recipient were subjected to antimicrobial susceptibility testing, and PCR to confirm blaCTX-M gene presence using the primers mentioned above. Plasmids were typed as well for both the donors and the transconjugants based on their incompatibility groups using the PCR based replicon typing scheme PBRT 2.0 Kit (Diatheva, Fano, Italy). Moreover, random amplified polymorphic DNA (RAPD) -PCR was performed for donor, transconjugants and recipients using RAPD-PCR kit (Amersham biosciences UK limited, England) to definitely distinguish transconjugants from donor strains.

3.5. Pulsed-Field Gel Electrophoresis (PFGE)

The Pulsed-Field Gel Electrophoresis (PFGE) was performed using XbaI restriction enzyme, the obtained genomic fragments were separated on a CHEF-DR II apparatus (Bio-Rad, Milan, Italy) for 22 h at 14°C. Bacteriophage λ concatenamers were used as DNA size markers. DNA restriction patterns of scanned gel pictures were interpreted following cluster analysis with the Fingerprinting II version 3.0 software (Bio-Rad) using the unweighted pair-group method with arithmetic averages (UPGMA). Only bands larger than 48 kb were considered for the analysis. The Dice correlation coefficient was used with a 1.0 % position tolerance to analyse the similarities of the banding patterns, and a similarity threshold of 90 % to define clusters. The restriction patterns of the genomic DNA from the isolates were analysed and interpreted according to the criteria of (19).

3.6. Phylogenetic groups investigation

Phylogenetic group typing was performed for the 16 ESβLs-producing *E. coli* isolates according to Clermont et al. using PCR assays targeting the genes *chuA*, *yjaA*, the DNA fragment TSPE4.C and *arpA* gene as described previously (20).

3.7. WGS and in silico analysis

Five out of the 16 ESβLs-producing *E. coli* strains were selected taking in consideration the highest level of antibiotic resistance and different phylogroups, and subjected to whole-genome sequencing for further investigation (Fig.1). Total DNA was extracted from pure overnight culture with E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Georgia, USA), following the manufacturer instructions. The DNA concentration was determined with Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and visualized on 0,8% agarose gel to check the DNA integrity. DNA was sequenced by Fasteris (Geneve, Switzerland) using Illumina Miseq platform (Illumina, Inc., San Diego, CA) with 300 paired-end run.

The quality of raw reads was evaluated with FastQC software (21). SPades tool on PATRIC website was used to perform the de novo assembly, discarding contigs with a length below 400bp (22) and contigs were annotated with Prokka v 1.13.3 with e-value cut-off default (23). In silico investigation of MultiLocus Sequence Type (MLST), serotype, fimH subtyping and phylogroup was investigated using MLST 2.0 for *E. coli* #1 (24), SerotypeFinder 2.0 (25), FimTyper-1.0 (26), using default threshold. ResFinder 4.1 (27), PlasmidFinder 2.1 (28) and VirulenceFinder 2.0 (29) were used to detect antimicrobial resistance genes, plasmid replicons and virulence factors.

Contigs harbouring ESβLs determinants were annotated with Prokka and the genetic environment of AMR genes were investigated using Geneious Prime v2021.2.2 and Isfinder (30). In particular, since $bla_{\text{CTX-15}}$ is known to be flanked by the transposon element ISEcp1 (AJ242809), a BLASTn search was performed on $bla_{\text{CTX-15}}$-positive genomes.

The phylogeny among wild boar strains was investigated analysing the pan genome with Roary (31). Then, the phylogenetic relationship between ST131 and ST10 strains from wild boars and other strains with the same sequence type and isolated from different sources was investigated.

We explored the genomes deposited in PATRIC and EnteroBase (32), for which information about the sequence type was available. The data about sequence type was confirmed with MLST 2.0 for *E. coli* #1 (24). Moreover, metadata about isolation source (human, animal, food), isolation country, and host health (where applicable) and ESβLs genes were also considered. All the selected genomes were re-analysed with ABRicate using ResFinder database (https://github.com/tseemann/abricate) and typing through SerotypeFinder.

Fifty-seven ST131 ESβLs-producing *E. coli* genomes with the mentioned features were obtained from PATRIC and EnteroBase (Table S1). When fastQ files were available, they were assembled with Spades.

FASTA files were re-annotated with Prokka v 1.13.3 with e-value cut-off default (23). The GFF files of all the downloaded ST131 and of WB 249 F2 strain were used for the pan and core genome analysis using Roary (31). The Newick file resulting from Roary analysis was uploaded in iTOL for viewing the relationship based on Single Nucleotide Polymorphism (SNP) detected in the core genome.

The same approach was adopted for the comparison between ST10 wild boar strains and genome sequences of ST10 strains isolated from different sources (Table S2). Genome sequences of ST10 EsβLs-producing *E. coli* were retrieved from PATRIC database. Sequences were screened confirming the ST group and a subset of 49 genomes were selected, considering the source (human, companion, and wild animal food) and the positivity for ESβLs genes.

4. RESULTS

4.1. Wild boars are carriers of ESβLs-producing and multi drug resistant (MDR) *E. coli*

Fourteen wild boars out of 60 (23.3%; 95% CI 14.4-35.4) were found to be positive for the presence of CTX-and CAZ-resistant *E. coli*. A total of 16 *E. coli* isolates were collected from the 14 animals from MLNs (5/60; 8.3%) and faeces (11/60; 18,3%) samples, with three wild boars positive both in MLNs and faeces. Positive wild boars were represented by 5 females (35.7%) and 9 males (64.3%) belonging to age class 0 (4/14; 26.6%) and age class 2 (10/29; 34.5%). All the 16 isolates were positive to the ESβLs-Confirm kit.

A wider evaluation of antimicrobial susceptibility profiles of the 16 ESβLs-producing *E. coli* showed 100% non-susceptibility to penicillins, third generation cephalosporins (3GCs) and fourth generation cephalosporins (4GCs), tetracyclines, monobactams, and tetracycline; 75% to trimethoprim/sulfamethoxazole, 37,5% to chloramphenicol, among fluoroquinolones 62,5% to ciprofloxacin and 31,25% to levofloxacin, against aminoglycosides 31,25% to tobramycin and 37,5% to gentamycin, and 18,75% to amoxicillin/clavulanate. All isolates resulted fully susceptible to carbapenems, amikacin, tigecycline, fosfomycin, piperacillin/tazobactam and colistin (Fig. 1).

Genotypic investigation was then used to determine the identity of genes conferring the phenotypic resistance. The presence of the $bla_{\text{CTX-M}}$-type genes was detected by microarray and confirmed using targeted PCR. $bla_{\text{CTX-M-245}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-1}}$, were found in 31.3% (n=5/16), 25% (n=4/16), 25% (n=4/16) strains, respectively. Among ESβLs-producing strains, fluoroquinolone resistant isolates (56.3%, n= 9/16) harboured either *qnrS* (55.6%, n=5/9), *aac(6')Ibcr* (33.3 %, n=3/9), or both *qnrS* and *aac(6')Ibcr* (1.1%, n=1/9) genes, while aminoglycoside resistant isolates (37.5%, n=6/16) harboured either *aadA* (33.3%, n=2/6), *aac(6')Ibcr* (50%, n=3/6), or *aadA* and *aac(6')Ibcr* (16.7%, 1/6) determinants (Fig.1).

4.2. Plasmid profile and conjugations results

The possible presence of plasmids was assessed for all 16 *E. coli* isolates. IncFII was the prevalent incompatibility group found in the isolates (n=6/16). Other groups detected included IncN (n=2/16), IncX1 (n=2/16), IncX3 (n=2/16), IncFIIS (n=2/16), IncFIIK (n=1/16), IncF (2/16), IncFIA (n=1/16), IncA/C (n=1/156), IncH12 (n=1/16), IncH1 (n=1/16), IncQ1 (1/16), and IncP1 (n=1/16). Two isolates resulted negative for all the Incompatibility groups searched. All strains were then subjected to conjugation experiments, and a lateral transfer of resistance genes was observed in 25% (n=4/16) of them. The resistance profiles of donors and transconjugants confirmed the lateral transfer of 3GCs resistance. PCR analysis confirmed the presence of a $bla_{\text{CTX-M}}$-type gene in all the transconjugants. Inc group plasmid and RAPD profile analysis performed on all the transconjugants confirmed that $bla_{\text{CTX-M}}$ gene was plasmid-encoded and that plasmids are mobilized by conjugation to the *E. coli* J53 or *E. coli* J62 recipient.

4.3. Molecular typing reveals high heterogeneity among ESβLs-producing *E. coli*

PFGE analysis of the 16 ESβLs-producing *E. coli* isolates showed a high clonal heterogeneity, revealing 14 different pulsotypes, even for the isolates recovered from MLNs and faeces of the same wild boar (Fig. 1). One *E. coli* isolate resulted not typable with this method.

The assignment to *E. coli* phylogenetic groups of the 16 isolates revealed that the 37,5% (n=6/16), all CTX-M-producers, belonged to phylogroup A; 37,5% (n=6/16) belonged to phylogroup B1; 12,5% (n=2/16) to phylogroup D; 6% (n=1/16) to phylogroup B1, and 6,25% (n=1/16) to phylogroup C (Fig. 1).

4.4. In-depth genomic characterization: typing, virulence, AMR and dissemination potential

Among the 16 ESβLs-producing *E. coli* strains, five of them were selected considering the resistance to high number of antimicrobials and the phylogroup, and sequenced for further deep characterization. As shown in Table 1, the *in silico* analysis evidenced 4 different Sequence Types (ST), and in particular two strains with ST10, one strain with ST131, one strain with ST46 and one strain with ST5051. The ST10 strains belonged to two different serogroups (O101:H9 and O127:H21), and ST131 strain belonged to O25:H4 serogroup; O8:H4 and O153:H9 were detected as serogroup of ST46 and ST505, respectively. While the ST131 strain showed the fimbrial variant fimH30, the other isolates harboured fimH54, except for WB221F2 which carries fimH34.

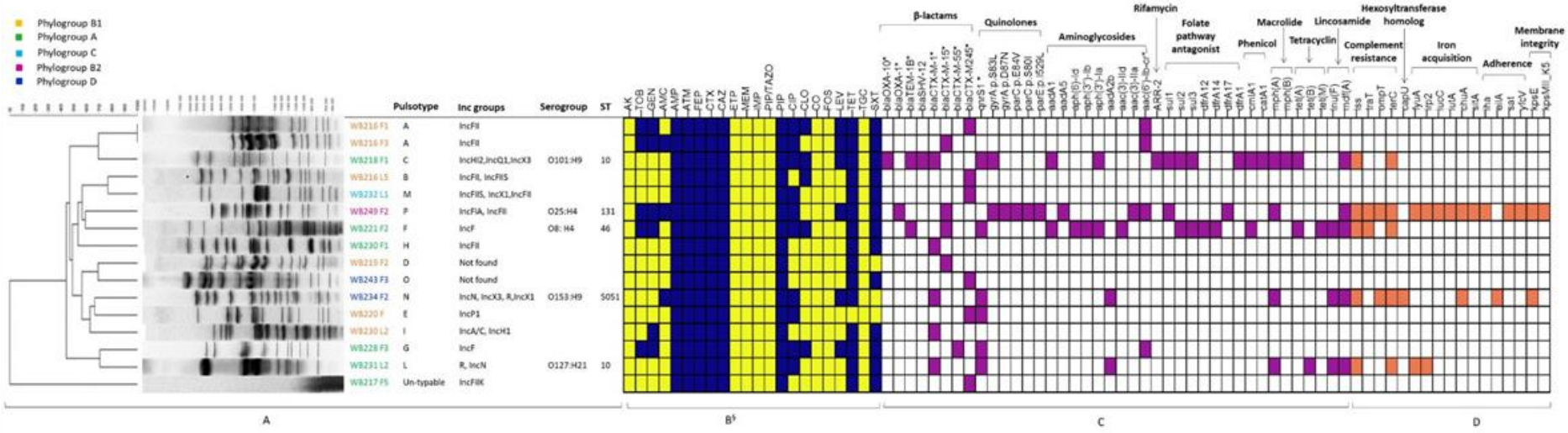

Figure 1. *The figure includes all the phenotypical and molecular characterization, including the analysis performed with WGS. The part (A) is related to the molecular typing by PFGE, with the identification of pulsotypes, phylogroups and plasmid replicons, for all the ESβLs-producing E. coli strains, while STs and serogroups are evidenced for the sequenced strains. The heatmap (B) shows the phenotypical resistance profile of all ESβLs-producing E. coli, where yellow identifies susceptible phenotype, while blue identifies the resistance phenotype. The molecular determinants find by PCR and genome analysis are evidenced in the heatmap in purple (C), while the virulence genes harbored by the sequenced strains are shown in orange (D). AMR genes and virulence factors are grouped by evidenced categories.*

§ AMC, amoxicillin/clavulanic acid; AMP, ampicillin; PIP, piperacillin; ATM, aztreonam; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; CXM, cefuroxime; CIP, ciprofloxacin; LEV, levofloxacin; TZP, piperacillin-tazobactam, AK. Amikacin; GM, gentamicin; TOB, tobramycin; CLO, chloramphenicol; ERT, ertapenem; ERT, ertapenem; IMI, imipenem; MEM, meropenem; TET, tetracycline; TMS, trimethoprim/sulfamethoxazole.

** Determinants detected by PCR.*

	WB218 F1	WB221 F2	WB231 L2	WB234 F2	WB249 F2
Genome size (bp)	4917558	4790178	4649545	5051588	5070855
N° contigs	113	164	74	78	73
MLST	10	46	10	5051	131
FimH group	54	34	54	54	30
Serogroup	O101: H9	O8: H4	O127:H21	O153:H9	O25:H4

Table 1*. Main genome features of five ESβLs-producing E. coli selected strains isolated from wild boars.*

The analysis of the genome led to a deep characterization of virulence and antimicrobial resistance content. The analysis of virulence profile evidenced the absence of Shiga-toxin genes (*stx1* and ***stx2***) and intimin gene (*eae*). Some other putative virulence factors were identified. In particular, WB249F2 shows a large collection of genes involved in adherence (*iha*, *yfcV*), complement resistance (*iss*, *traT* and *ompT*), iron acquisition (*iucC*, *iutA*, *chuA*, *fyuA*, *irp2*) and membrane integrity (*kpsE*, *kpsMII_K5*). The other strains showed a less enriched panel of virulence factors, some of whom were in common with WB249F2 strain. Differently from all other strains, WB234F2 carried hexosyltransferase homolog gene (*capU*) and Salmonella *hilA* homolog (*eilA*).

A more accurate and comprehensive typing of antimicrobial determinants was assessed using the WGS analysis (Fig. 1). Among ESβLs genes, the five sequenced wild boar strains were confirmed to carry the same determinants found by molecular analysis and moreover was able to detect other genes. Indeed, the screening of genome revealed that WB218F1 strain was a carrier of also $bla_{\text{TEM-1B}}$ and $bla_{\text{SHV-12}}$ genes. It is interesting to notice that while all strains were positive for $bla_{\text{CTX-M}}$ genes, the WB218F1carried also all the other EsβLs determinants.

Genome analysis of other resistance genes confirmed the most resistance phenotypes against other antimicrobials, identifying determinants directly related to the phenotype. Indeed, WB218F1 showed a point mutation in *gyrA* gene (S83L) linked to the fluoroquinolone phenotype, while the resistance of WB249F2 strain was due to mutations in *gyrA* (S83L and D87N) gene associated with the *parC* (E84V, S80I) and *parE* (I529L) mutations.

WB218F1 and WB221F2 were positive for *cmlA1* gene, responsible for the resistance against chloramphenicol together with *catA1* and *flor1*, respectively. The analysis revealed that strains (WB221F2 and WB249F2) phenotypically resistant to aminoglycosides like gentamicin and tobramycin carried not only *aac(6')-Ib-cr* genes but also other genes belonging to the aminoglycoside-(3)-N-acetyl-transferase family, such as *aac(3)-IId* and *aac(3)-IIa*. From a phenotypic point of view, all five strains resulted resistant to tetracycline; this evidence was confirmed at genome level only for three strains, which carried *tet(A)*, *tet(B)* and *tet(M)*. The tetracycline resistance for WB234F2 was related to the presence of *mdf(A)*, a multidrug resistance determinant, while in the case of WB249F2 strain no genes were identified. Moreover, the analysis lead to identify the presence of other genes linked to the resistance to trimethoprim-sulfamethoxazole such as *dfrA1*, *dfrA12*, *dfrA17*, *sul3*, *sul2*, *sul1* in

WB218F1, WB221F2 and WB249F2 genomes. No gene mutations usually linked to levofloxacin were identified, due to the absence of correlation in the ResFinder/PointFinder database (33). The possibility of AMR genes to be mobilized between strains is directly linked to their localization on mobile genetic elements, i.e. plasmids and transposons. The genome of five strains was screened for plasmid replicons; moreover, this information was linked to the investigation of genome makeup around AMR genes in order to study the possibility of gene sharing through mobile elements. The analysis with PlasmidFinder matched with the results of PCR (Table 2).

The replicon position at contig level was compared to the position of ESBL determinants, assuming that, if AMR genes were carried by plasmid, they would be found near the replicons. The analysis revealed that none of them were located on the same contig of plasmid replicon.

strain	Plasmid replicon	Contig position	Contig length (bp)
WB218 F1	IncHI2	46	10181
	IncHI2 2	42	22264
	IncX3	37	35167
WB221 F2	IncFIB	92	4852
		12	104785
WB231 L2	IncN	26	31042
	IncR	32	15115
WB234 F2	IncN	28	31174
	IncR	33	11243
	IncX1	25	45110
WB249 F2	IncFIA	29	17426
	IncFII	35	8144

Table 2*. Identification of plasmid replicons in the sequenced ESβLs-producing E. coli strains. For each strain, the Incompatibility group (plasmid replicon) is highlighted with the related number of contig on which the replicon was found, and the length of contig.*

In particular, the beta-lactamase genes are located on short contigs (Fig.2), preventing a complete view of the genomic makeup around them. Anyway, the annotation of these contigs was performed and evidenced that *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{SHV-12}}$ genes of WB218F1 strain were surrounded by IS6 and Tn3 family transposase (Fig. 2), differently from *bla*$_{\text{OXA-1}}$ which was not enclosed by any transposable elements. The same situation was detected for other strains. *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1B}}$ genes of WB221F2 strain were bracketed with Tn3-like transposase and TnpA, as well as *bla*$_{\text{CTX-M-15}}$ of WB249F2; no mobile genetic elements were detected near *bla*$_{\text{CTX-M-1}}$ gene of WB231L2 and WB234F2 strains. Moreover, the Blastn search evidenced the presence of the transposon ISEcp1 near the *bla*$_{\text{CTX-M-15}}$ genes in WB231L2 and WB249F2.

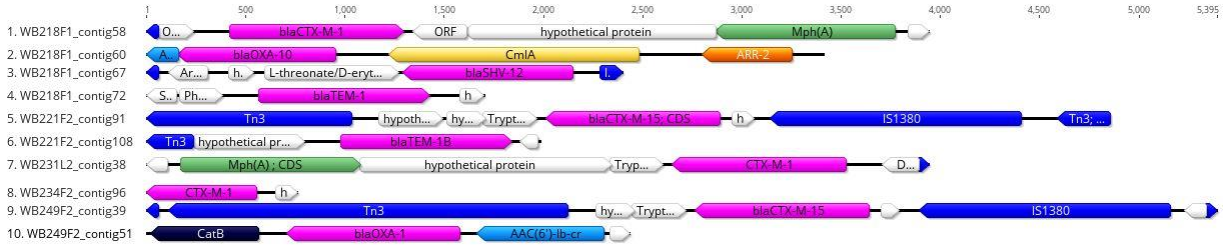

Figure 2*. Genomic context of ESβLs genes in wild boar isolates. Each line represents a contig harboring the ESβLs determinants and it is identified by the name of strain and number of contig. bla$_{CTX}$, bla$_{OXA}$, bla$_{TEM}$ and bla$_{SHV}$ genes are colored in purple, while the mobile elements are evidenced in blue. Green, yellow, orange and light blue indicate other AMR genes, while hypothetical or other proteins are white.*

4.5. Phylogenetic insight of wild boar ESβLs-producing *E. coli* and the correlation with ST131 and ST10 ESβLs-producing *E. coli* isolated from different sources

In order to understand how strains were genetically correlated, the pan-genome analysis was carried out. The phylogenetic relationship between wild boar strains is evidenced in the Figure 3.

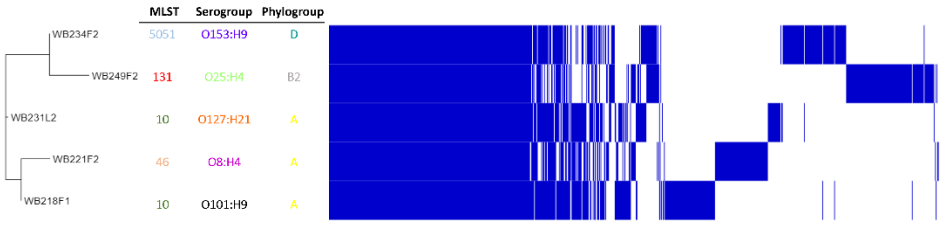

Figure 3. *Pangenome analysis of ESβLs-producing E. coli isolated from wild boars. The figure evidence, on the left, the phylogenetic tree, built on SNPs of core genes. Identification of Sequence Type (MLST), serogroup and phylogroup are also indicated.*

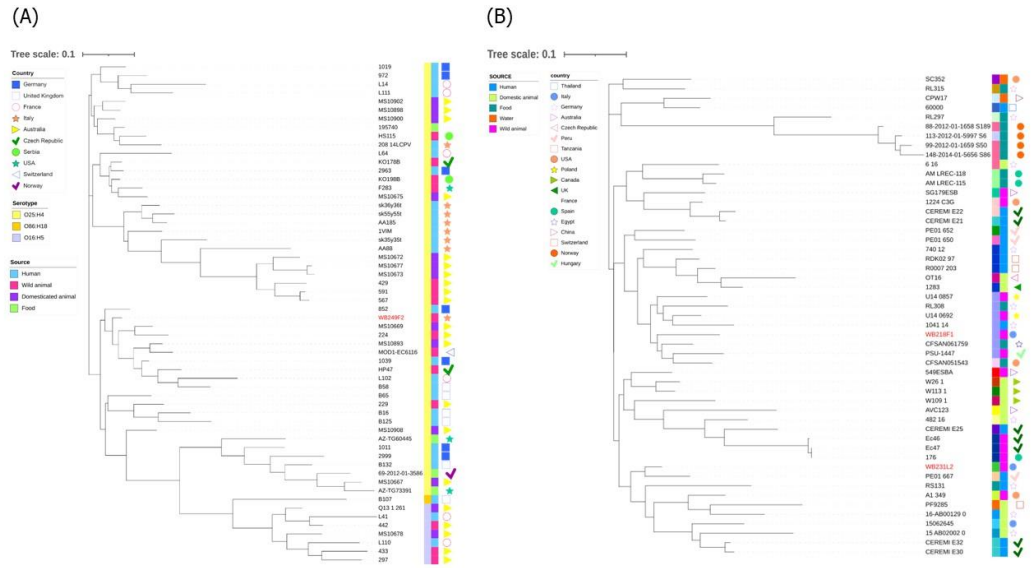

Figure 4. *Phylogenetic tree built on the SNPs of core genes in the ST131 (A) and ST10 (B) ESβLs-producing E. coli strains analyzed. (A) WB249F2, the ST131 strain isolated from wild boar, was compared with other 57 strains selected by isolation source and country, and (B) the same analysis was performed comparing WB218F1 and WB231L2 with other 49 strains, chosen following the same criteria. The wild boar isolates are highlighted in red in order to better evidence them. Color-coding is used to report the serotype (first line), isolation source (second line), while isolation countries are represented by different symbols.*

All the strains shared 3325 genes (45% of pangenome), which represent the core genome; the remaining non-core 4085 genes were divided into 1316 accessory genes (18% of pangenome) shared by 2 to 4 strains and 2769 unique genes (cloud genes; 37% of pangenome). The phylogenetic tree built on SNPs of core genes divided strains in three groups, showing high degree of heterogeneity inside and reflecting the difference in the genetic composition in term of virulence and antimicrobial resistance profile, ST and serogroup.

Then, wild boar isolates with ST131 and ST10 were considered for further investigation, with the aim to compare them with other strains with the same ST, isolated from different sources, namely human, food, wild and domestic animals. While the phylogenetic tree shows a clear clusterization of analysed depending on serotype (Fig. 4A), including the wild boar ST131 strain in the O25:H4 group, it evidences also a widely distribution of genomes belonging to different source and country. The same situation is observed for the comparison among the ST10 strains (Fig. 4B). Although for the ST10 genomes a more heterogeneous situation was observed regarding the serogroups, the relationship between wild boar isolates and other strains seems do not follow a strict criterion. Interestingly, the 2 strains are located far from each other, placing WB231L2 near human and domestic animals, while the WB218F1 near to food and wild animal isolates.

5. DISCUSSION

The epidemiology of ESβLs-producing microorganisms is quite complicated, including geographical areas, hospitals, communities, hosts, as well as various reservoirs represented by the environment (soil and water), farmed animals, wild animals, and pets. Transmission from food and water, or from direct contact (person-to-person), represents the final step of ESβLs-producing bacteria epidemiology (34). The role of wild animals, including wild boars, as maintenance hosts of ESβLs-producing *E. coli* has already been assessed worldwide (35–37), with some phylogenetic groups showing clinical relevance (38)). The acquisition of ESβLs-producing *E. coli* by wild boars has been proposed to be related to their proximity to human and other animals' habitats, as well as by their omnivorous habits which may give the animals the possibility to eat human food waste (39), as well as carrions and vegetables contaminated by animal manure.The major aim of our study was the genomic characterization of EsβLs-producing *E. coli* isolated from wild boars in terms of antimicrobial resistance, virulence, mobile genetic elements and epidemiology (phylogroups and MLSTs). Since these animals were hunted in mountain areas with limited anthropic influence but they can also move in wider area, they can be considered as indicators of the environmental load of AMR. The comparison with similar studies performed in Northern Italy confirms the high prevalence of ESβLs-producing *E. coli*, as 15.96% of ESβLs/AmpC *E. coli*-positive wild boars hunted during 2017-2020 in Lombardy region (40). In our study, where the overall prevalence of ESβLs-*E. coli* carriers reached 23.3%, the adults were more frequently positive (35.5%) if compared to the young (26.6%) and the subadults (0.0%), probably because they could move in wider territories and come in contact with different ESβLs sources. While young wild boars (0-12 months) were highly exposed to the colonization due to their impaired immune system, subadults (12-24 months) were probably more reactive in eliminating the microorganisms from the gut. In the present study, all the suspected ESβLs-producing *E. coli* showed a MDR profile, with a high percentage (>60%) of fluoroquinolones non-susceptibility level; this represents a particular concern, as this antibiotic class is frequently employed to treat clinical infections. The ESβLs of CTX-M-type resulted the most spread 3GCs/4GCs hydrolysing enzymes among the collected isolates. Although this fact is already established and reported in the literature, it is nevertheless interesting to note that the allelic variants identified included the hyper-represented and globally widespread $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$. We also found the $bla_{\text{CTX-M-245}}$ variant, never described before in Italy, nor in Europe. It was reported only once from a *Serratia marscescens* isolate collected from a blood sample of a neonate in Iraq (41). This finding suggests that human-animal proximity could also lead to the exchange and circulation of under-detected variants of resistance genes. The transferability of some resistance plasmids, mainly those ESβLs-harbouring, is a further cause for concern. In addition to the ESβLs encoding genes, the isolates studied also showed fluoroquinolone and aminoglycoside resistance genes, as *qnrS*, *aac(6')Ibcr* and *aadA* alone or in combination. Interestingly, the molecular typing showed a very high heterogeneity in terms of clonal lineages; isolates collected from different sites

(MLNs and feces) of the same wild boar resulted not clonally related to each other. Among the strains isolated from wild boars, five were selected in order to be deeper characterized from a genomic point of view. Interestingly, the *in silico* genome analysis highlighted a pandemic clone ST131 isolated from wild boar feces, belonging to the O25:H4 serogroup and B2 phylogroup. This clone is recognized as highly virulent group of ExPEC (extraintestinal pathogenic *E. coli*), which is responsible for urinary tract infections, bacteremia, urinary sepsis and neonatal sepsis. This strain harbors genomic features typical of ESβLs ExPEC, since it shows *bla*$_{\text{CTX-M-15}}$ gene, other than *bla*$_{\text{OXA-1}}$, and a virulence pattern which includes genes responsible for an improved adherence and ability to survive in the human body (42). Since the worldwide diffusion of ST131 clones in humans and in wildlife as well (43–45), it is not surprising to find this ST in wild boars (46,47). This fact is supported by the phylogenetic analysis which places WB249F2 near human isolates, and near wild and domestic animals as well. While ST131 clone commonly shows a rich set of virulence genes (48,49), we found other STs with a less extensive virulence pattern. Notably, we isolated two ST10 strains, which are frequently responsible for AMR-resistant human infection (50,51). The same sequence type was detected in wild boars in Germany (47), and it is known to be common especially in poultry (52,53). From the analysis of core genome SNPs, the correlation between ST10 wild boar strains and other strains isolated from different origin, especially human and food, from one side, and human and domestic animal from the other side, has been highlighted. These results confirm again the heterogeneity observed among the isolates of this study, suggesting that wild boars could be a carrier and spreader of any type of ESβLs-producing *E. coli* clones. The other STs detected seem not to be widespread. Indeed, the ST46 was found rarely in water in Bangladesh (54) and Chinese companion animals (55), while no other authors detected the ST5051 clone. The capability of antimicrobial resistance genes to be shared with other bacteria in the same niche represents a major threat. This possibility is strictly connected to the genome organization and genomic feature of *E. coli*, in particular with mobile genetic elements, namely plasmids, transposons and insertion sequences. With the aim to investigate if the AMR genes could be transferred to other strains, the genomes of 5 strains were screened. Overall, the ESβLs genes were found to be surrounded by different type of transposons, despite the assembly procedure was not able to reconstruct in a definitive way the genomic context. Since the mobility of AMR can be driven not only by the entire plasmid but also by transposable elements, we cannot exclude the exchange of AMR gene through this mechanism. Further analysis should be performed using long-read sequencing, as suggested by others (56).

6. CONCLUSION

The main finding of our study is the detection of the ExPEC clone ST131 and the ESβLs-MDR ST10 *E. coli* isolates in a restricted wild boar population living in Northern Italy, thus suggesting a circulation

of human pathogenic *E. coli* strains among wildlife. Since the concept of One Health is based on the connection between humans, animals, plants, and environmental health, especially in geographically close systems such as the area of the study, we can assess that wild animals should be included in each AMR surveillance programs. Their role as indicators of the environmental load of AMR is especially evident in our study, since all ESβLs-producing *E. coli* strains showed a MDR phenotype, including resistance to highly important antimicrobials.

Given the importance of genome analyses to recognize the different *E. coli* clones and trace their occurrence in humans as well as in other species, we strongly suggest the WGS as the most effective tool to investigate ESβLs-producing *E. coli*.

7. DATA AVAILABILITY STATEMENT

Sequencing data were submitted to the Sequence Read Archive (SRA) under the Project ID PRJNA759409.

8. AUTHOR CONTRIBUTIONS

SB collected samples and performed the strains isolation. AA, AP, AM, FM and SB performed phenotypic and molecular analysis. CC and GM carried out bioinformatic analysis. AP, CC and SB wrote the manuscript. PSC, RM, and SB supervised the experiment design and all the analysis and contributed to the manuscript revision. All authors have read and approved the final draft of the manuscript.

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10. SUPPLEMENTARY MATERIALS

Strain	Accession number	Serotype	Country	Source	***Bla*$_{tem}$**	***Bla*$_{ctx}$**	***Bla*$_{oxa}$**	***Bla*$_{kpc}$**	***Bla*$_{cmy}$**
1011	GCA_001647335.1	O25:H4	Germany	H	blaTEM-235				
1019	GCA_001647315.1	O25:H4	Germany	H	blaTEM-1				
1039	GCA_001647325.1	O25:H4	Germany	H	blaTEM-1		bla OXA-1		
2963	GCA_001561105.1	O25:H4	Germany	H	blaTEM-1	blaCTX-M-27			
2999	GCA_001561195.1	O25:H4	Germany	H	blaTEM-235				
852	GCA_001555495.1	O25:H4	Germany	H	blaTEM-1		bla OXA-1		
972	GCA_001555635.1	O25:H4	Germany	H	blaTEM-1				
B107	GCA_001561735.1	O86:H18	UK	H	blaTEM-1				
B125	GCA_001561785.1	O25:H4	UK	H		blaCTX-M-15			
B132	GCA_001561705.1	O25:H4	UK	H		blaCTX-M-1			
B16	GCA_001561275.1	O25:H4	UK	H		blaCTX-M-15	bla OXA-1		
B58	GCA_001561495.1	O25:H4	UK	H	blaTEM-1				
B65	GCA_001561285.1	O25:H4	UK	H	blaTEM-1	blaCTX-M-15	bla OXA-1		

L102	GCA_003427875.1	O25:H4	France	H			bla OXA-1	
L110	GCA_003427575.1	O16:H5	France	H	blaTEM-1			
L111	GCA_003427815.1	O25:H4	France	H	blaTEM-1	blaCTX-M-1		
L14	GCA_003428115.1	O25:H4	France	H		blaCTX-M-1		
L41	GCA_003428105.1	O16:H5	France	H	blaTEM-1	blaCTX-M-1		
L64	GCA_003427895.1	O25:H4	France	H	blaTEM-30			
sk35y35t	ERX4530492	O25:H4	Italy	H	blaTEM-90	blaCTX-M-15	blaOXA-1 and 9	blaKPC-2
sk36y36t	ERX4530493	O25:H4	Italy	H	blaTEM-122	blaCTX-M-27	blaOXA-9	blaKPC-2
sk55y55t	ERX4530512	O25:H4	Italy	H	blaTEM-79	blaCTX-M-27	blaOXA-9	blaKPC-3
208_14LCPV	data unpublished	O25:H4	Italy	H	blaTEM-1			
AA185	data unpublished	O25:H4	Italy	H	blaTEM-1	blaCTX-M-27	blaOXA-9	blaKPC-3
AA88	data unpublished	O25:H4	Italy	H	blaTEM-1		blaOXA-9	blaKPC-3
1VIM	data unpublished	O25:H4	Italy	H	blaTEM-1 and 79		blaOXA-9	blaKPC-2
MS10667	GCA_018728485.1	O25:H4	Australia	CLF	blaTEM-1			
MS10669	GCA_018728495.1	O25:H4	Australia	CLF			bla OXA-1	
MS10672	GCA_018728425.1	O25:H4	Australia	CLF		blaCTX-M-15	bla OXA-1	
MS10673	GCA_018728365.1	O25:H4	Australia	CLF		blaCTX-M-15	bla OXA-1	
MS10675	GCA_018728385.1	O25:H4	Australia	FC		blaCTX-M-27		
MS10677	GCA_018728345.1	O25:H4	Australia	CLF		blaCTX-M-15	bla OXA-1	
MS10678	GCA_018728325.1	O16:H5	Australia	FC		blaCTX-M-14		
MS10893	GCA_018728245.1	O25:H4	Australia	CLF	blaTEM-1	blaCTX-M-15		
MS10898	GCA_018728265.1	O25:H4	Australia	CLF	blaTEM-1			
MS10900	GCA_018728225.1	O25:H4	Australia	CLF	blaTEM-1			
MS10902	GCA_018728155.1	O25:H4	Australia	CLF	blaTEM-1	bla CTX-M-14		
MS10908	GCA_018728145.1	O25:H4	Australia	CLF	blaTEM-1			
Q13_1_261	GCA_018728525.1	O16:H5	Australia	CLF	blaTEM-1			
591	SRR7724635	O25:H4	Australia	WA		blaCTX-M-15	blaOXA-1	

567	SRR7724756	O25:H4	Australia	WA		blaCTX-M-15	blaOXA-1		
429	SRR7724649	O25:H4	Australia	WA		blaCTX-M-15	blaOXA-1		
442	SRR7724742	O16:H5	Australia	WA	blaTEM-1	blaCTX-M-15			
433	SRR7724657	O16:H5	Australia	WA		blaCTX-M-15			
297	SRR7724771	O16:H5	Australia	WA		blaCTX-M-15			
229	SRR7724629	O25:H4	Australia	WA		blaCTX-M-15	blaOXA-1		
224	SRR7724623	O25:H4	Australia	WA	blaTEM-1	blaCTX-M-15			
HP47	GCA_001561865.1	O25:H4	Czech Republic	WA	blaTEM-1	blaCTX-M-15	blaOXA-1		
KO178B	GCA_001637865.1	O25:H4	Czech Republic	WA		blaCTX-M-27			
KO198B	GCA_001638005.1	O25:H4	Serbia	WA		blaCTX-M-27			
F283	GCA_001561845.1	O25:H4	USA	WA		blaCTX-M-27			
HS115	GCA_001561895.1	O25:H4	Serbia	WA	blaTEM-1	blaCTX-M-15	blaOXA-1		
MOD1-EC6116	GCA_002463555.1	O25:H4	Switzerland	WA	blaTEM-1	blaCTX-M-15			
AZ-TG73391	SRR1220791	O25:H4	USA	F	blaTEM-1				
AZ-TG60445	SRR1178255	O25:H4	USA	F	blaTEM-1				
69-2012-01-3586	ERR4769384	O25:H4	Norway	F					blaCMY-2
195740	SRR5031226	O25:H4	UK	F	blaTEM-1				

Supplementary Table S1*. List of ST131 ESβL-producing E.coli genomes used for the comparison with WB249F2, the ST131 strain isolated from wild boars. Genomes were selected by country (UK: United Kingdom; US: United States), source (H: Homo sapiens; CLF: Canus lumpus familiaris; FC: Felis catus; F: food; WA: Wild Animal), and ESβL genes variants.*

STRAIN	**ACCESSION NUMBER**	**serotype**	**country**	**source**	**bla-TEM**	**bla-CTX**	**bla-OXA**	**bla-SHV**	**bla-CMY**	**blaNDM**
60000	GCA_014117545.1	O nd:H12	Thailand	H	blaTEM-1	blaCTX-M-3	blaOXA-1	blaSHV-12		blaNDM-1
15062645	GCA_018193775.1	O117:H10	Italy	DA	blaTEM-150	blaCTX-M-1				
1041_14	GCA_014691585.1	O101:H9	Germany	H	blaTEM-154				blaCMY-2	
15_AB02002_0	PRJEB21546	O8:H32	Germany	DA	blaTEM-150					

16-AB00129_0	PRJEB21546	O50:H27	Germany	DA		blaCTX-M-1		
482_16	PRJNA514245	O132:H28	Germany	DA	blaTEM-235			blaCMY-2
6_16	PRJNA514245	O nd:H4	Germany	DA	blaTEM-1			blaCMY-2
740_12	GCA_014691485.1	O101:H10	Germany	H	blaTEM-1			blaCMY-2
AVC123	GCA_003338865.1	O132:H21	Australia	DA	blaTEM-1			
OT16	GCA_003591315.1	O nd:H10	Czech Republic	DA	blaTEM-34			blaCMY-2
PE01_650	GCA_013425265.1	O166:H4	Peru	H	blaTEM-1			
PE01_652	GCA_013425145.1	O99:H33	Peru	H	blaTEM-1			
PE01_667	GCA_013424045.1	H21	Peru	H	blaTEM-150			
R0007_203	GCA_900239835.1	O101:H10	Tanzania	H	blaTEM-235		blaOXA-1	
RDK02_97	GCA_900239925.1	O101:H10	Tanzania	H			blaOXA-1	
RL297	GCA_014683685.1	O23:H32	Germany	F				blaCMY-132
RL308	GCA_014688505.1	O101:H9	Germany	F				blaCMY-2
RL315	GCA_014691445.1	O2:H2	Germany	F	blaTEM-1			blaCMY-2
RS131	GCA_014687265.1	H27	Germany	H	blaTEM-1	blaCTX-M-65	blaOXA-10 and 1	blaCMY-2
SC352	GCA_003358245.1	O127:H16	USA	W	blaTEM-1			
U14_0692	GCA_016697145.1	O101:H9	Polands	WA	blaTEM-154			
U14_0857	GCA_016697225.1	O101:H9	Polands	WA	blaTEM-1			
W109_1	GCA_011600965.1	O50:H4	Canada	DA		blaCTX-M-1		
W113_1	GCA_011601165.1	O28ac/O42:H37	Canada	DA		blaCTX-M-1		
W26_1	GCA_011600865.1	O109:H32	Canada	DA		blaCTX-M-1		
1283	GCA_002310735.1	O101:H10	UK	DA				blaCMY-2

CEREMI_E21	SAMEA4853120	O73:H31	France	H					blaCMY-138
CEREMI_E22	SAMEA4853121	O8:H17	France	H					blaCMY-138
CEREMI_E25	SAMEA4853122	O71:H48	France	H	blaTEM-1	blaCTX-M-1			
CEREMI_E30	SAMEA4853123	O73:H31	France	H		blaCTX-M-1			
CEREMI_E32	SAMEA4853124	O73:H31	France	H		blaCTX-M-1			blaCMY-138
176	GCA_012618795.1	O16:H48	Spain	WA	blaTEM-122				
1224_C3G	GCA_014689345.1	O8:H17	USA	WA				blaSHV-12	
549ESBA	GCA_014158385.1	O26:H32	Australia	WA	blaTEM-1B	blaCTX-M-15			
AM_LREC-115	GCA_017783665.1	O153:H10	Spain	WA	blaTEM-1A	blaCTX-M-32			
CFSAN061759	PRJNA230969	O101:H9	Egypt	WA	blaTEM-1B	blaCTX-M-15			
CPW17	PRJNA408214	O86:H12	China	F	blaTEM-1B	blaCTX-M-14	blaOXA-1		
Ec47	PRJNA705836	O16:H48	France	F	blaTEM-122				
PF9285	GCA_004771235.1	O117:H4	Switzerland	W	blaTEM-1B				
CFSAN051543	GCA_012664925.1	O9:H9	USA	WA	blaTEM-1B				
AM_LREC-118	GCA_017783505.1	O153:H10	Spain	DA	blaTEM-1A	blaCTX-M-32			
113-2012-01-5997_S6	SAMEA7483614	O125:H4	Norway	F					blaCMY-2
88-2012-01-1658_S189	SAMEA7483587	O nd:H4	Norway	F					
99-2012-01-1659_S50	SAMEA7483588	O nd:H4	Norway	F					

148-2014-01-5656_S86	SAMEA7483649	O nd:H4	Norway	F		
A1_349	SAMN14534474	O45:H45	USA	F		blaCMY-2
Ec46	SAMN18104154	O16:H48	France	F	blaTEM-1B	
PSU-1447	SAMN10315796	O101:H9	Hungary	WA	blaTEM-1A	
SG179ESB	GCA_014779015.1	O92:H33	Australia	WA		blaCTX-M-15

Supplementary table S2*. List of T10 ESβL-producing E.coli genomes used for the comparison with WB218 and WB231, the ST10 strain isolated from wild boars. Genomes were selected by country (UK: United Kingdom; US: United States), source (H: Human; DA: domestic animal; F: food; WA: Wild Animal; W: water), and ESβL genes.*

CHAPTER 4

Unrestricted Gene Exchange: Transfer of Tn*916*-Mediated Tetracycline Resistance Gene in Food, Animals, and the Environment.

Unrestricted Gene Exchange: Transfer of Tn*916*-Mediated Tetracycline Resistance Gene in Food, Animals, and the Environment.

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1. Abstract

The dissemination of antimicrobial resistance in the environments, including foods, pose a rising public health concern at global level. In this study, we applied a gene exchange model involving the transfer of the tetracycline resistance gene *tet*(M) carried by the conjugative transposon Tn*916* from the commensal *Enterococcus faecium* UC7251 to two foodborne pathogens, *Listeria monocytogenes* DSM 15675 and Scott A, to evaluate the rate of horizontal gene transfer in different segments of the food system. We investigated this gene transfer event in fermented food models of cheese and sausage, where the latter showed higher conjugation frequencies. Then, *Galleria mellonella,* a model of terrestrial invertebrates, was utilized in *in vivo* experiments with a conjugation frequency of 10^{-5} transconjugant/donor for both strains. The transfer dynamics was examined after biofilm formation on environmental polyethylene microplastic particles, in a marine model containing *Mytilus galloprovincialis*. The *tet*(M) transfer occurred in mussels, exhibiting conjugation rates of 10^{-6} transconjugant/donor. No conjugation was observed in the absence of microplastics. In our study, both *Listeria* strains were able to acquire *tet*(M), suggesting that Tn*916* was able to spread genes of concern without the need for the recipient strains to carry a conjugation machinery. The presence of enterococci resistant to antimicrobial , particularly *E. faecium*, in the food chain, warrants closer monitoring due to their fundamental role in the spreading of antibiotic resistance in *L. monocytogenes* and other foodborne pathogens.

2. Introduction

The emergence of antimicrobial resistance (AMR) is a global threat to human and animal health, occurring in all environments, where microbial communities are exposed to anthropogenic use of antimicrobials, such as clinical settings and farm animals [1], [2]. The excessive and inappropriate use of antimicrobial compounds has led to the escalation of AMR, amplifying its propagation across diverse microbial ecosystems including open environments and the food systems [3].

The primary cause for the dissemination of antimicrobial resistance genes (ARGs) is horizontal gene transfer (HGT) via mobile genetic elements (MGEs), such as plasmids and transposons [4]. Particularly conjugative transposons, are mobile genetic elements that often carry antibiotic resistance genes, pathogenicity genes and metabolic capabilities. These elements play a role in bacterial evolution by imparting particular phenotypes to host cells[5]. HGT can occur in complex matrices like environments, animal guts and food, where high-density bacterial populations contribute to its occurrence [6], [7]. Moreover, in these environments, non-pathogenic and commensal AMR bacteria act as reservoirs of ARGs, which can be transferred to pathogenic bacteria, limiting the effectiveness of commonly used antimicrobial drugs and making infections more difficult to treat. Hence, the food system has been proposed as one of the key factors in the spread of AMR across the One-health continuum, from the environment and animals to humans [1]. The number of studies that address this issue considering different sections of the food chain is still limited. The propagation of ARGs in fermented food has been studied in the last years, highlighting that, commensal bacteria harbouring resistance determinants and

naturally contaminating meat and milk, are able to deliver them to the microbiota sharing the same niche [8]. Some examples were reported in fermented meats for coagulase negative staphylococci [9], *Lactobacillaceae* spp. strains [10] and *Enterococcus faecium* and *Enterococcus faecalis* [11]. In dairy products, different species of lactic acid bacteria (LAB) and enterococci have been identified to harbour ARGs, indicating the potential transmission of these genes between them. However, only a limited number of researchers have directly investigated the HGT of resistance determinants in cheese [12], [13].

The AMR spread has been also observed in environmental niches and, among them, the seawater aquatic environment. Thus, it was shown to act as a reservoir for resistant bacteria originating from human and animal sources, contributing to the dissemination of ARGs [14]. Mussels, a common organism found in seawater, accumulate bacteria from the surrounding water through their suspension filtration process, making them valuable biomonitoring tools for assessing AMR in aquatic environments [15]. Additionally, the pollutants of aquatic environments such as microplastics have raised concerns about the dissemination of ARGs as they provide surfaces for biofilm formation, creating favourable conditions for HGT [16]–[18]. The limitation of *in vitro* conditions can be overcome by utilizing *in vivo* models such as animal models using insects, that are useful for preliminary tests prior to proceeding with mammalian studies [19]. The assessment of *in vivo* HGT between different species and genera can be effectively evaluated using animal models, such as the utilization of wax moth *Galleria mellonella* larvae, characterised by an innate immune system closely analogous to that of mammals, offering a closer resemblance to the human situation compared to *in vitro* assays. In particular *Galleria mellonella* larvae has become a surrogate organism for the study HGT *in vivo* and thus a suitable preliminary model before conducting mammalian studies, greatly reducing costs and testing time[19], [20]. Furthermore, terrestrial invertebrates can act as environmental reservoirs for antimicrobial resistance genes. Understanding how these genes spread within invertebrate populations may shed light on potential transmission routes to other organisms and environments[21].

Several non-pathogenic foodborne bacteria, such as *E. faecium,* have raised public health concerns for representing AMR reservoirs contributing to their spread to humans through the food chain, animals and the environment [11], [22], [23]. *E. faecium* is remarkably prone to develop AMR compared to other bacteria with particular attention to tetracycline, one of the most widely used antimicrobials in humans and animal production [24]. Moreover, it has been reported that tetracycline resistance in *E. faecium* can be acquired and transferred through transposons, especially the conjugative transposon Tn*916* [25]. A previous study reported the successful passage of this determinant from *E. faecium* of fermented meat origin to *Listeria monocytogenes in vitro* [26]. The latter, an important foodborne pathogen, frequently detected in cheese, meat and seafood products, has been shown to be able to acquire or transfer AMR genes from MGEs including, either *in vitro* or within the intestinal tract [27].

In a previous study, the multidrug resistant *E. faecium* strain UC7251 isolated from fermented dry sausage, was shown to harbour several MGEs with a capacity to transfer AMR genes intra- and inter-genera [24]. Specifically, UC7251 carries the conjugative transposon Tn*916* inside an integrative conjugative element

(ICE) region, and a 192 kb plasmid harbouring genes coding for resistance to tetracycline, macrolides and aminoglycosides. This strain showed a high ability to withstand environmental stresses, being resistant up to 10% NaCl and to heavy metals such as copper, zinc and cadmium.

The objective of this study is to utilize an established gene exchange model involving the transfer of the Tn*916*-tetracycline resistance gene *tet*(M) from the commensal *E. faecium* UC7251 to the foodborne pathogen *L. monocytogenes* to assess the rate of HGT in various sections of the food chain. Specifically, we investigated HGT in cheese and sausages food models, that more closely simulate conditions present in the food matrix, such as free water content, nutrient and salt concentration. Moreover westudied the transfer dynamics using the bivalve *Mytilus galloprovincialis* as a representative model for seafood, examining also the influence of the environmental contaminant polyethylene microplastics on HGT. Additionally, the *Galleria mellonella* larvae, commonly utilized as host in microbiological studies, was employed as a terrestrial invertebrate model to further investigate HGT dynamics in the environment and along the food chain.

3. Materials and methods

3.1 Bacterial strains and bioinformatic analyses

E. faecium UC7251, isolated from a dry fermented sausage and previously characterized [24], was used as the donor strain and cultured in Brain Heart Infusion (BHI) (Oxoid) supplemented with 10 μg/ml of tetracycline (Sigma Aldrich) and 50 μg/ml of erythromycin (Sigma Aldrich) and incubated at 37 °C overnight. *Listeria monocytogenes* DSM 15675 and Scott A, cultivated in BHI and incubated at 37°C overnight, were used as recipient strains. *E. faecium* and *L. monocytogenes* genomes were downloaded from NCBI (Genbank accession UC7251: GCA_000411655.2; Scott A: GCA_009866905.1; DSM 15675: GCA_002156185.1) and analysed for the presence of MGE with ICEfinder [28].

3.2 Filter mating and plate mating

In vitro conjugation experiments were carried out with two different approaches, filter mating and plate mating. The filter mating assay was adjusted from a previously reported method[29] with some modifications. Briefly, the donor and recipient strains were grown overnight in BHI broth. Absorbance was measured and corrected to OD600 = 1.0 ± 0.05 for each culture. Recipient and donor strains were centrifuged at 4100 × g for 10 min) and pellets were washed twice with 1 mL of Phosphate Buffered Saline (PBS) 0.1 M, and finally re-suspended in 1 mL of PBS 0.1 M. Then, 500 μL of donor and 500 μL recipient cultures were mixed well. The resulting mixture was centrifuged and the pellet re-suspended in 200 μL of PBS 0.1 M. For conjugation, a Durapore® membrane filter (Merck Millipore, Burlington, MA, USA) was placed onto BHI Agar and the conjugation mixture was transferred to the centre of the filter. The plate was incubated for 72 hours at 37 °C. After 24,48 and 72 three replicates of filters were transferred in 2 mL of 0.9% NaCl solution and homogenized. The conjugation mixture was serially diluted and plated on Chromogenic Listeria Agar Base (ALOA) (Oxoid) supplemented with tetracycline

(10μg/ml) to select transconjugants and, on Slanetz and Barley (SB) (Oxoid) supplemented with tetracycline (10μg/ml) and erythromycin (50μg/ml) to select the donor. The plates were incubated at 37°C for 24 hours in aerobic conditions. The plate mating was carried out as previously described by [30] with some minor modifications. After an overnight incubation, 100 μL of donor cells were mixed with 900 μL of recipient cells in a 1.5 mL microtube. The mixture was then centrifuged at room temperature for 15 min at 3000 × g and, the pellet was re-suspended in 0.1 ml of BHI. The obtained suspension was plated on the surface of BHI agar and incubated for 72 h at 37°C under aerobic conditions. After the three incubation period cells were retrieved from the plate and re-suspended in 1 ml of saline solution, serially diluted, plated and incubated as describe above.

3.3 Gene exchange in meat-based and cheese-based food models.

The conjugation experiment was performed in two types of food models, one reproducing the chemical characteristics of fermented meat and the other representing dairy products. To prepare the meat model, 60 g of pork mixture was finely minced and pasteurized for 30 min at 65°C. At the end of the heat treatment, 12 ml of sterile H_2O and 180 ml of 2% Agar-H_2O solution are added to the mixture and thoroughly blended to maximize nutrient dissolution. The mixture was filtered using sterile gauze to eliminate large meat particles. Following the recipe used in the industrial production, glucose (Carlo Erba) (0.5 % of total weight), NaCl (Carlo Erba) (3 % of total weight) and $NaNO_3$ (Carlo Erba) (150 ppm) were added. For the cheese model, semi-hard cheese was finely minced and mixed with 50 ml of Agar-H_2O 1%. The mixture was pasteurized for 30 min at 65°C. At the end of the heat treatment, 1.9 ml of sterile 20% lactose solution was added.

For the conjugation experiments, 100 μL of an overnight culture of donor and recipient strains previously washed three times with saline solution, were placed at the bottom of a square petri plate well (Thermo Fisher Scientific), covered by 4 mL of the meat agar mixture or the cheese agar mixture and carefully mixed with a sterile loop. The plate was incubated at 37°C in aerobic conditions for 72 h. Three replicates of meat and cheese agar mixture respectively were taken after 24, 48 and 72 h of incubation, and serially diluted with saline solution and plated as previously described. The plates were incubated at 37°C for 24 hours in aerobic conditions.

3.4 *In vivo* gene exchange in *Galleria mellonella* larvae.

The evaluation of *in vivo* gene transfer in the animal model *Galleria mellonella* was performed by injecting the larvae with the conjugation mixture. This method was conducted as described by Morgan et al. (2014) with modifications to adapt it to the conjugation test [31]. Briefly, donor and recipient bacterial overnight cultures were pelleted by centrifugation (4000 x g for 10 min) and washed twice in Phosphate Buffer Solution (PBS) (0.1 M) and finally re-suspended in PBS, normalized using optical density (OD_{600}=0.7). Three independent biological replicates of 10 larvae were infected with 10 μl of donor and recipient strains in a ratio 1:1, by injection with a Hamilton syringe (26 gauge) via the last right proleg. A negative control with only PBS was also included. Larvae were incubated at 37° C in the dark for 24 h. After the

incubation period larvae of each replicate were homogenized, serially diluted and plated onto selective ALOA and SB supplemented with antibiotics as described above.

3.5 *In vivo* gene exchange in *Mytilus galloprovincialis* in aquatic environmental model.

The assessment of AMR gene transfer was studied *in vivo* in mussels with or without microplastics in the controlled aquatic environment composed o artificial seawater ASTM D1141-98 (ThermoFisher Scientific) in a 60 L fish tank (Radys) with water recycling and oxygenation. Seventy undamaged and live mussels were added to the fish tank with microplastic particles covered by biofilm produced separately with donor and recipient strains. The biofilm formation was carried out as previously described by others with some modifications [32]. Five grams of Polyethylene molecular weight 35000 (PE 35000) (Sigma Aldrich) pre-sterilized with ethanol 95% for 24 h were placed into different 250 ml glass flasks with 100 ml of BHI. Each glass flask was individually inoculated with 1.5 ml of overnight bacterial cultures, washed with saline solution, of UC7251, DSM 15675 and Scott A, and then incubated at 37°C without agitation for 6 days. The biofilm determination was performed as previously described by Hchaichi et al. (2020) [32]. Then, the microplastics were recovered aseptically, washed three times with distilled water. The obtained microplastics sample with the donor and recipient strains biofilm were added into the fish tank.

The same experiment was carried out without microplastics. An overnight culture of the donor and recipient strains was washed three times, with saline solution, and inoculated into the fish tank in a ratio 1:1000. After 4 and 7 days, three replicates of 10 bivalves were separated from the shells, re-suspended in 90 ml of saline solution, and homogenized in a stomacher three times for 5 min. The obtained mixtures were serially diluted and plated to select transconjugants as detailed above. At the same time points, the conjugation frequency was assessed analysing three replicates of 10 ml of seawater. A negative control in which neither planktonic cells nor biofilm-covered microplastics was added. The water temperature was maintained at 15° C in all experiments.

3.6 Transconjugants confirmation, statistical analysis and safety hazards

The obtained transconjugants were confirmed using gene specific PCR with primers *tet*(M) as previously described [24]. The conjugation frequencies was calculated as previously described [33]. The experiments were performed in at least three independent replicates. Statistical analysis was performed using Past4.06b. Conjugation frequency were analysed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test ($p \leq 0.05$). Experiments were conducted in a BL2 (Biosafety Level 2) Bacteriology Laboratory, in accordance with the WHO guidelines [34].

4 Results

4.1 Mobile genetic elements in UC7251 and *Listeria monocytogene*s strains.

For the purpose of assessing the effect of MGE in the efficiency of gene exchange in different models, we selected two different strains of *Listeria monocytogene*s on the basis of the presence of MGEs. The genome investigation for MGEs in strain DSM 15675 resulted in the absence of any type of MGE, whereas Scott A presented an integrative conjugative element of 58 kb (2375271-2433301bp) containing the conjugative transposon Tn*5422*. The latter carried heavy metal resistance genes for cadmium and arsenic. A more detailed genomic screening of UC7251 revealed that the integrative conjugative element carrying Tn*916* is classified as a conjugative system MPF type FA containing a relaxase, type 4 coupling protein (T4CP) and a VirB4 type four secretion system (T4SS), with accessory profile genes containing orf13-14-15-16-17a-17b-19 and 23.

4.2 Conjugation *in vitro* by filter and plate mating

The conjugation experiments carried out *in vitro* confirmed the capability of the donor strain UC7251 to transfer the *tet*(M) resistance gene to both the recipient strains *L. monocytogenes* DSM 15675 and Scott A. For both recipient strains, an increasing number of transconjugants were observed throughout the analysis period with the highest conjugation frequency at 72 h, for both approaches. For DSM 15675, conjugation frequencies in plate and filter mating reached values of 10^{-5} transconjugants/donors (T/D). While Scott A showed a T/D of 10^{-5} in plate mating and 10^{-6} for filter mating. No significant differences were observed between tested strains and mating techniques at the three times of analysis. Conjugation frequency and the relative statistical analysis, for both *L. monocytogenes* strains were reported in Table 1S and 2S.

4.3 Conjugation in food models

Experiments on food models were developed to estimate the conjugation frequency in conditions mimicking fermented meat and cheese. Table 3S and 4S shows the conjugation frequencies obtained with food models and the relative statistical analysis at the three different testing times for the two *L. monocytogenes* strains. The analysis highlighted the increase of conjugation rates with the increase of mating time between donor and recipient. For both strains in the meat model at 72h, a significant increase in conjugation rate was observed during the analysis period. In the meat model, statistically significant higher conjugation frequencies when compared to the cheese model were observed in experiments with strain Scott A . In the cheese model at 72h, conjugation frequencies obtained with Scott A were statistically lower than those with DSM 15675 (Fig.1a and 1b).

4.4 Conjugation in vivo *using* Galleria mellonella

After observing high conjugation frequencies of gene exchange in food matrices, our aim was to evaluate this event in an animal model that represented the dissemination of ARGs in the environment. For this scope, *Galleria mellonella* was used to evaluate the capability of the donor UC7251 to transfer the Tn*916* to the recipient strains of *L. monocytogenes* in an *in vivo* terrestrial invertebrate model. (Fig.1c). Table 5S shows the conjugation frequencies obtained and the relative statistical analysis. The frequency obtained

in vivo did not present statistically significant differences between the two recipient strains with a T/D rate of 2.5 $x10^{-5}$ (-4.59 Log) and 2.4 $x10^{-5}$ (-4.62 Log), for DSM 15675 and Scott A, respectively. No tetracycline resistant *Listeria* spp. was detected in the negative control**,** with a limit of detection of 10 CFU/ml.

4.5 Conjugation in aquatic environmental model

The HGT of the *tet*(M) gene was assessed in an aquatic model that incorporates live mussels and microplastics, reproducing the marine environment. The controlled aquatic environment was recreated to evaluate the ability of *M. galloprovincialis,* a filter-feeding aquatic organisms, to act as a host HGT in bacterial communities in the presence or absence of microplastics. First, the ability of the three strains, the *E. faecium* UC7251 and the two recipients *L. monocytogenes* DSM 15675 and Scott A, to form biofilms on PE microparticles was tested. All these strains were able to form biofilms on the PE surface, with the highest formation on the sixth day of incubation (data not shown). No conjugation events were found when the donor and recipients were inoculated as planktonic cells in water in absence of microplastics (fig. 1d and 1e), with a limit of detection of 10 transconjugants/ml. When PE microparticles separately colonised by the donor or recipient strain biofilms were added to the seawater, HGT was detected after 4 and 7 days of incubation, with a final frequency of transfer of 2.49 x 10^{-7} (-6.60 Log) and 2.13 x 10^{-7} (-6.68 Log) for DSM 15675 and Scott A, respectively. Statistically significant higher frequencies of *tet*(M) gene exchange were found in mussels, when the biofilm covered microplastics, were added into the fish tank. Conjugation frequencies in mussels significantly increased over time, from 4 to 7 days. On day 7, DSM 15675 presented a T/D rate of 6.79 x 10^{-6} (-5.17 Log) and Scott A of 8.76 x 10^{-6} (-5.06 Log), and no significant differences were reported between the two tested strains as shown in (fig. 1d and 1e).

Table 6S and 7S show the conjugation frequency and the related statistical analysis. No tetracycline resistant *Listeria* spp. was detected in the negative control**,** with a limit of detection of 10 CFU/ml.

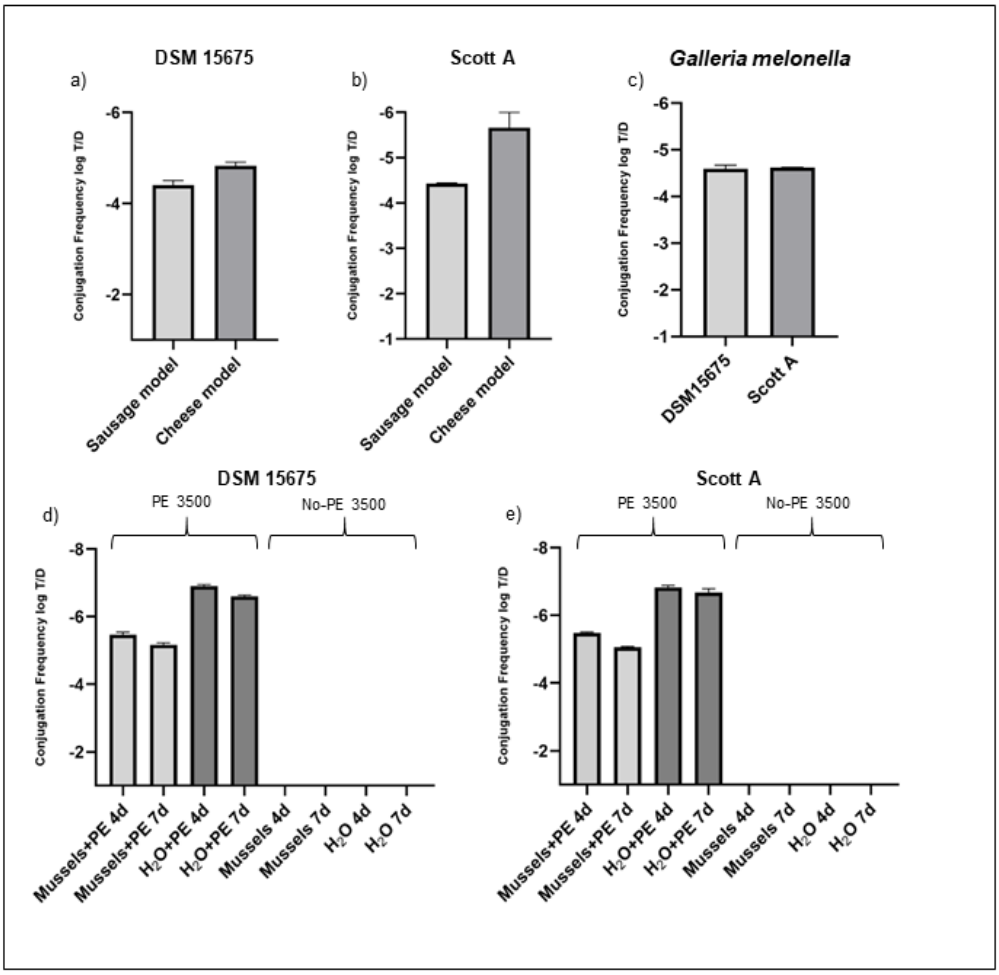

Figure 1. *Conjugation frequencies expressed as Log of T/D (Transconjugants/Donors) detected in: a) meat and cheese model after 72 h, for* L. monocytogenes *DSM 15675; b) meat and cheese model after 72 h, for* L. monocytogenes *Scott A; c)* in vivo *using* G. mellonella *larvae; d)* in vivo *in* M. galloprovincialis *and in seawater with (PE) and without microplastics in aquatic environmental model for* L. monocytogenes *DSM 15675; e)* in vivo *in* M. galloprovincialis *and in seawater with (PE) and without microplastics in aquatic environmental model for* L. monocytogenes *Scott A. The figure shows the average values from three independent replicates. Error bars represent standard error of the mean.*

5. Discussion

The worldwide spread of AMR is rapidly increasing, posing a constant threat to humans, animals and the environment [35]. The objective of this study was to employ a gene exchange model for assessing the HGT rate at different stages of the food chain. Specifically, we focused on the transfer of the tetracycline resistance gene *tet*(M), carried by Tn*916*, from the commensal bacterium *E. faecium* UC7251 to the foodborne pathogen *L. monocytogenes*. In particular, we selected the strain DSM 15675, lacking mobile genetic elements, and Scott A, containing the conjugative transposon Tn*5422* [36], in order to assess if the presence of conjugation machinery, encoded by transposons, within the recipient would affect the capability of Tn*916* to transfer *tet*(M). An initial screening with filter and plate mating was carried out with the aim to optimize experimental conditions and to test the tetracycline conjugation capacity to *L. monocytogenes* strains DSM 15675 and Scott A. Our results confirm a similar conjugation frequency (T/D) of 10^{-5} obtained by previous studies where the transfer of tetracycline resistance from *E. faecium* to *L. monocytogenes* was reported in filter, liquid and plate mating [37], [38].

We focused on exploring HGT in the context of fermented food models, namely cheese and sausages, which are matrices where *Enterococcus* and *Listeria* species are frequently found and isolated [39], [40]. Our results showed that in the sausage model, the horizontal gene transfer frequency was not statistically significant between the two strains DSM 15675 and Scott A, with values of 10^{-5}. Differently, in the cheese model, DSM 15675 presented a T/D value of 10^{-5}, significantly higher than Scott A with T/D value of 10^{-6}. The distinct sources origins of *L. monocytogenes* DSM 15675, isolated from soft cheese, and *L. monocytogenes* ScottA, isolated from a patient affected by listeriosis in Massachusetts due to consumption of pasteurized milk, may offer insights into their varying degrees of adaptation to the dairy environment. This differential adaptation could potentially account for the higher occurrence of conjugation events observed in the context of this food matrix. Taken together, all the experiments *in vitro* with plate and filter mating and with food models showed that the presence of Tn*5422* in strain Scott A did not affect the conjugation capacity of UC7251-Tn*916* to transfer *tet*(M) efficiently. A few studies have explored the capacity of transfer of antibiotic resistance genes during the ripening of fermented sausages [41], [42], supporting the fact that during this process, the genetic transfer is promoted by the bacterial contact. Interestingly, other studies have performed *in situ* experiments on salmon and fermented chicken sausage, where Tn*916*-carried *tet*(M) was transferred to *E. faecalis* [13], [43]. Moreover, other studies have also explored the capacity of Tn*916*-mediated transfer of *tet*(M) from *L. monocytogenes* and *E. faecalis in situ* on the surface of cheese, with results comparable to ours [12], [44]. The transfer of the conjugative transposon Tn*916* in cheese is relevant, as enterococci could come in close contact with *Listeria* spp. and other bacteria during cheese ripening and storage [45]. The acquisition of ARGs by *L. monocytogenes* or other pathogens is of considerable concern as it is associated with a decrease in the efficacy of available treatments [46].

To gain further insights into HGT dynamics in both the environment and the food chain, we utilized *Galleria mellonella* larvae as a terrestrial invertebrate model [19], [47], [48]. The conjugation frequencies obtained *in vivo* through injection, reached T/D values of 2.58 x 10^{-5} (-4.59 Log) and 2.41 x 10^{-5} (-4.62 Log) for *L. monocytogenes* DSM 15675 and Scott A, respectively. In a previous study, Göttig and colleagues investigated the HGT of gene OXA-48 using *G. mellonella*, finding higher transmission frequencies in *in vivo* than *in vitro* mating experiments [49]. Our study proves that *G. mellonella* larvae act as a host for HGT of the *tet*(M) resistance gene and that such exchange may occur within their gastrointestinal tract under laboratory conditions. The high gene transfer rate could be due to the ability of trasconjugant *L. monocytogenes,* carrying the *tet(*M) gene from conjugation, to adhere and enter to the intestinal tract cells of larvae [50], [51], resulting in a concentration effect. The high capacity of *L. monocytogenes* to tolerate environmental stresses, and in particular acid stresses, and to create biofilm are of crucial importance for the colonization of the gastrointestinal tract, thus acting as a possible reservoir of ARGs that can be transferred to other resident or transient gut bacteria[52].

Additionally, we investigated the dynamics of gene transfer using the bivalve species *Mytilus galloprovincialis* as a representative model for seafood and seawater environment. Moreover, we examined the potential impact of environmental contaminant polyethylene microplastics on HGT processes. In recent decades, microplastic pollution has become one of the most pressing environmental challenges, with a particular emphasis on aquatic environments [53]. Mussels are now recognized as a useful indicator of microplastic pollution in marine environments given their wide distribution in ecological niches and their high susceptibility to microplastic accumulation [15]. Previous studies have assessed the presence of pathogenic and AMR bacteria in bivalve mussels and seafood, raising a problem that needs to be monitored [54]–[57]. This led us to evaluate the ability of UC7251 to transfer the *tet*(M) to *L. monocytogenes* in a complex aquatic model, including mussels, as filtering organisms in which gene exchange could take place [58]–[60], and to assess the role of microplastics on the frequency of horizontal gene exchange [61]. Our results support that UC7251 is unable to transfer resistance to tetracycline in the aquatic environment model in absence of microplastics. Thus, no transconjugants were detected in the experiments without the presence of the PE particles either in the seawater or in the mussels. Differently, when polyethylene (PE) microplastics, pre-colonized by *L. monocytogenes* and *E. faecium* separately, were introduced into the seawater, conjugation took place at a frequency of 3 x 10^{-6} (-6.6 Log) for both recipient strains on day four. In this case, mussels acted as biological amplifier of the horizontal gene exchange, since the frequencies were significantly higher than those observed in water. The HGT detected in mussels increased significantly over time, with a T/D of 6.8 x 10^{-6} (-5.17 Log) and 8.8 x 10^{-6} (-5.06 Log) for *L. monocytogenes* DSM 15675 and Scott A on day seven, respectively. Although mussels are rather used as bioindicator for seawater and not commonly used as a model for assessing HGT, previous studies support the fact that ARG transfer can occur in the aquatic ecosystem and in the marine wildlife acting as a reservoir of antibiotic resistance [62]–[65]. For instance, Guglielmetti and colleagues isolated *L. garvieae* from salmonoid fish and demonstrated *in vitro* the transfer capacity of *tet*(S) to three

different strains of *L. monocytogenes* [66]. In another study, a *in vivo* approach was applied to assess the ARGs conjugal transfer in the intestines of zebra fish, highlighting that the fish gut promote the HGT [67]. Another study demonstrated the transfer of vancomycin resistance in *E. faecalis* in a similar acquatic model using zebra mussels *Dreissena polymorpha* [68]. By exposing the zebra mussels to specific concentrations of strains of *E. faecalis* (donor isolate MF06036^{VanA} and recipient isolate MW01105^{Rif}), the gene transfer occurred at a maximal transfer efficiency of 10^{-6} (T/D) for mussel parts (visceral mass, shell and gills) [68]. In contrast to previous findings where conjugation was observed in animal models without the presence of microplastics, our results demonstrate that this phenomenon exclusively occurs when both the donor and recipient are carried by microplastics. Our study highlighted the role of mussels as filtering organism involved in facilitating the emergence and spread of AMR in the natural environment.

Moreover, the role of microplastics as a reservoir and vehicle for the spread of ARG has been observed in different studies [69], [70]. In agreement with our results, other studies support the involvement of microplastics in promoting biofilm formation and thus significantly increasing ARG transfer, compared to water samples without microplastics[71]–[73]. Indeed, Arias-Andres and colleagues discovered a significantly higher frequency of plasmid transfer among bacteria associated with microplastics compared to free-living bacteria or natural aggregates in an aquatic environment. [17], [74].

In various *in vitro*, food, animal, and environmental models, it has been observed that the presence of conjugation machinery in the recipient strains does not have a significant impact on the gene transfer capacity of Tn*916* of UC7251. This is primarily attributed to the existence of a Type IV secretion system (T4SS) and a relaxase in donor cells that promotes the transfer of genes to the recipient cell without requiring these proteins to be present in the recipient cell itself [75].

Overall, we observed notable rates of gene transfer in various ecological contexts and food-related scenarios. This highlights the concerning potential for antibiotic resistance dissemination from a One-Health perspective. Additionally, we developed models to assess the HGT between commensal and foodborne pathogenic bacteria within the food system. Furthermore, our research draws attention to the role of environmental pollutants, such as microplastics in marine bivalves, in facilitating the spread of transposon-coded antimicrobial resistance among food-associated bacteria. The dissemination of ARGs in the food chain is a complex issue with broad implications for the health of humans, animals, and the environment. These findings underline the need for a holistic and collaborative One Health approach to implement effective mitigation strategies to combat the widespread and occurrence of antibiotic resistance, including responsible antibiotic use in agriculture, surveillance, and interventions aimed at safeguarding public health and ecosystem integrity.

6. Author Contributions

G.M.: Methodology, Formal Analysis, Investigation, Writing-Original Draft Preparation

M.V.B.D.: Formal Analysis, Investigation, Writing-Original Draft Preparation.

C.C.: Formal Analysis, Writing-Review and Editing.

D.B.: Writing-Review and Editing

P.S.C.: Conceptualisation, Writing-Review and Editing, Validation, Visualisation, Supervision, Project Administration.

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8. Data Availability Statement

The genomic data used in this study (Accession numbers of *E. faecium* UC7251: GCA_000411655.2; *L. monocytogenes* Scott A: GCA_009866905.1; *L. monocytogenes* DSM 15675: GCA_002156185.1) can be found in online repositories (NCBI, https://www.ncbi.nlm.nih.gov/genome/?term=). The conjugation frequencies and the relative ANOVA results can be found in the supplementary from Table 1S to Table 7S.

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10. SUPPLEMENTARY MATERIALS

Table 1S. Conjugation frequency in plate and filter mating.

		Conjugation Frequency [Log(T/D)]			Standard deviation		
Strain	**Type**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
	filter mating	-5.18	-5.24	-4.65	0.07	0.03	0.04
***Listeria monocytogenes* DSM 15675**	**plate mating**	-5.48	-5.44	-5.00	0.21	0.12	0.11
	filter mating	-6.34	-6.20	-5.10	0.27	0.53	0.10
***Listeria monocytogenes* Scott A**	**plate mating**	-5.60	-5.22	-5.00	0.19	0.23	0.65

Table 2S. ANOVA of conjugation frequencies in plate and filter mating.

			***Listeria monocytogenes* DSM 15675**						***Listeria monocytogenes* Scott A**					
			filter mating			**plate mating**			**filter mating**			**plate mating**		
Strain	**Type**	**Time of analysis**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
***Listeria monocytogenes* DSM 15675**	**filter matin g**	**24 h**		1,0 E+0 0	5,0 E-01	9,4 E-01	9,9 E-01	1,0 E+0 0	9,0 E-04	8,1 E-04	1,0 E+0 0	7,2 E-01	1,0 E+0 0	1,0 E+0 0
	filter matin g	**48 h**	4,0 E-01		3,4 E-01	9,9 E-01	1,0 E+0 0	1,0 E+0 0	1,8 E-03	1,6 E-03	1,0 E+0 0	8,6 E-01	1,0 E+0 0	1,0 E+0 0
	filter matin g	**72 h**	3,3 E+0 0	3,7 E+0 0		3,6 E-02	6,4 E-02	9,0 E-01	4,0 E-06	3,6 E-06	7,0 E-01	1,1 E-02	3,1 E-01	4,1 E-01
	plate matin g	**24 h**	2,1 E+0 0	1,7 E+0 0	5,3 E+0 0		1,0 E+0 0	5,7 E-01	2,8 E-02	2,5 E-02	8,2 E-01	1,0 E+0 0	9,9 E-01	9,7 E-01
	plate matin g	**48 h**	1,7 E+0 0	1,3 E+0 0	4,9 E+0 0	3,8 E-01		7,4 E-01	1,5 E-02	1,4 E-02	9,2 E-01	1,0 E+0 0	1,0 E+0 0	9,9 E-01
	plate matin g	**72 h**	1,0 E+0 0	1,4 E+0 0	2,2 E+0 0	3,1 E+0 0	2,7 E+0 0		1,5 E-04	1,4 E-04	1,0 E+0 0	2,9 E-01	9,9 E-01	1,0 E+0 0
	filter matin g	**24 h**	7,5 E+0 0	7,1 E+0 0	1,1 E+0 0	5,5 E+0 0	5,9 E+0 0	8,6 E+0 0		1,0 E+0 0	4,1 E-04	8,2 E-02	2,1 E-03	1,3 E-03
	filter matin g	**48 h**	7,6 E+0 0	7,2 E+0 0	1,1 E+0 0	5,5 E+0 0	5,9 E+0 0	8,6 E+0 0	6,6 E-02		3,7 E-04	7,4 E-02	1,8 E-03	1,1 E-03
***Listeria monocytogenes* Scott A**	**filter matin g**	**72 h**	4,6 E-01	8,6 E-01	2,8 E+0 0	2,5 E+0 0	2,1 E+0 0	5,9 E-01	8,0 E+0 0	8,1 E+0 0		5,2 E-01	1,0 E+0 0	1,0 E+0 0
	plate matin g	**24 h**	2,8 E+0 0	2,4 E+0 0	6,0 E+0 0	7,1 E-01	1,1 E+0 0	3,8 E+0 0	4,8 E+0 0	4,8 E+0 0	3,2 E+0 0		8,9 E-01	8,0 E+0 0
	plate matin g	**48 h**	4,9 E-01	8,8 E-02	3,8 E+0 0	1,6 E+0 0	1,2 E+0 0	1,5 E+0 0	7,0 E+0 0	7,1 E+0 0	9,5 E-01	2,3 E+0 0		1,0 E+0 0
	plate matin g	**72 h**	2,0 E-01	2,0 E-01	3,5 E+0 0	1,8 E+0 0	1,5 E+0 0	1,2 E+0 0	7,3 E+0 0	7,4 E+0 0	6,6 E-01	2,6 E+0 0	2,8 E-01	

Table 3S. *Conjugation frequency in sausage and cheese models.*

Strain	Type	Conjugation Frequency [Log(T/D)]			Standard deviation		
		24 h	48 h	72 h	24 h	48 h	72 h
Listeria monocytogenes **DSM 15675**	**sausage model**	-5.24	-5.15	-4.40	0.08	0.10	0.10
	cheese model	-6.44	-5.89	-4.82	0.19	0.47	0.08
Listeria monocytogenes **Scott A**	**sausage model**	-6.22	-5.78	-4.43	0.26	0.35	0.02
	cheese model	-6.30	-6.30	-5.49	0.22	0.20	0.45

Table 4S. *ANOVA of conjugation frequencies in sausage and cheese models.*

			Listeria monocytogenes DSM 15675						*Listeria monocytogenes* Scott A					
			sausage model			**cheese model**			**sausage model**			**cheese model**		
Strain	**Type**	**Time**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
Listeria monocytogenes DSM 15675	**sausage model**	**24 h**		1,00E+00	1,73E-02	2,20E-04	3,57E-02	6,69E-01	2,05E-03	1,80E-01	2,17E-02	9,98E-04	1,09E-03	6,90E-01
	sausage model	**48 h**	6,48E-01		4,80E-02	7,39E-05	1,26E-02	9,02E-01	6,80E-04	7,37E-02	5,95E-02	3,31E-04	3,62E-04	4,09E-01
	sausage model	**72 h**	5,77E+00	5,13E+00		2,93E-08	2,52E-06	6,59E-01	1,87E-07	1,51E-05	1,00E+00	1,02E-07	1,10E-07	1,46E-04
	cheese model	**24 h**	8,36E+00	9,00E+00	1,41E+01		5,97E-01	2,03E-06	9,98E-01	1,87E-01	3,56E-08	1,00E+00	1,00E+00	2,55E-02
	cheese model	**48 h**	5,32E+00	5,97E+00	1,11E+01	3,04E+00		2,90E-04	9,82E-01	1,00E+00	3,14E-06	9,20E-01	9,32E-01	8,22E-01
	cheese model	**72 h**	2,88E+00	2,23E+00	2,90E+00	1,12E+01	8,19E+00		1,63E-05	1,98E-03	7,20E-01	8,23E-06	8,96E-06	1,94E-02
Listeria monocytogenes Scott A	**sausage model**	**24 h**	7,05E+00	7,69E+00	1,28E+01	1,31E+00	1,73E+00	9,92E+00		6,79E-01	2,30E-07	1,00E+00	1,00E+00	1,74E-01
	sausage model	**48 h**	4,19E+00	4,84E+00	9,97E+00	4,16E+00	1,13E+00	7,07E+00	2,85E+00		1,91E-05	4,93E-01	5,16E-01	9,97E-01
	sausage model	**72 h**	5,63E+00	4,98E+00	1,42E-01	1,40E+01	1,10E+01	2,76E+00	1,27E+01	9,82E+00		1,24E-07	1,34E-07	1,85E-04
	cheese model	**24 h**	7,47E+00	8,12E+00	1,32E+01	8,89E-01	2,15E+00	1,03E+01	4,22E-01	3,28E+00	1,31E+01		1,00E+00	9,82E-02

cheese model	**48 h**	7,42 E+00	8,06 E+00	1,32 E+01	9,41 E-01	2,10 E+00	1,03E +01	3,70E-01	3,22E +00	1,31 E+01	5,28 E-02	1,06 E-01
cheese model	**72 h**	2,83 E+00	3,47 E+00	8,60 E+00	5,53 E+00	2,49 E+00	5,70E +00	4,22E +00	1,37E +00	8,46 E+00	4,64 E+00	4,59 E+00

Table 5S. *Conjugation frequency in vivo using Galleria mellonella.*

	Listeria monocytogenes DSM 15675		*Listeria monocytogenes* Scott A	
	24 h	**Standard deviation**	**24 h**	**Standard deviation**
conjugation by injection	-4.59	0.08	-4.62	0.01

ANOVA		
***Listeria monocytogenes* DSM 15675**		1
***Listeria monocytogenes* Scott A**	1,09E-02	

Table 6S. *Conjugation frequency in aquatic model using Mytilus galloprovincialis.*

			Conjugation Frequency [Log(T/D*100)]		Standard deviation	
			4 days	**7 days**	**4 days**	**7 days**
Listeria monocytogenes DSM 15675	**PE 3500**	***M. galloprovincialis***	-5.48	-5.17	0.07	0.06
		H2O	-6.91	-6.60	0.05	0.04
	No-PE 3500	***M. galloprovincialis***	ND	ND	ND	ND
		H2O	ND	ND	ND	ND
Listeria monocytogenes Scott A	**PE 3500**	***M. galloprovincialis***	-5.49	-5.06	0.02	0.03
		H2O	-6.83	-6.68	0.06	0.11
	No-PE 3500	***M. galloprovincialis***	ND	ND	ND	ND
		H2O	ND	ND	ND	ND

Table 7S. *ANOVA of conjugation frequencies in Aquatic model Mytilus galloprovincialis.*

			***Listeria monocytogenes* DSM 15675**				***Listeria monocytogenes* Scott A**			
			M. galloprovincialis		**H2O**		***M. galloprovincialis***		**H2O**	
			4 days	**7 days**	**4 days**	**7 days**	**4 days**	**7 days**	**4 days**	**7 days**
***Listeria monocytogenes* DSM 15675**	***M. galloprovincialis***	**4 days**		2,70E-04	6,56E-14	3,96E-12	1,00E +00	5,78E-06	1,70E-13	1,43E-12
		7 days	8,76E +00		2,19E-14	6,31E-14	1,68E-04	3,57E-01	2,27E-14	3,35E-14
	H2O	**4 days**	4,09E +01	4,96E +01		3,09E-04	7,59E-14	2,14E-14	7,77E-01	5,64E-03
		7 days	3,22E +01	4,10E +01	8,66E +00		4,70E-12	2,80E-14	5,73E-03	7,81E-01

***Listeria monocytogenes* Scott A**	***M. galloprovincialis***	**4 days**	3,73E-01	9,14E+00	4,05E+01	3,19E+01		3,86E-06	2,04E-13	1,71E-12
		7 days	1,20E+01	3,23E+00	5,29E+01	4,42E+01	1,24E+01		2,18E-14	2,35E-14
	H2O	**4 days**	3,87E+01	4,75E+01	2,18E+00	6,48E+00	3,83E+01	5,07E+01		1,05E-01
		7 days	3,44E+01	4,32E+01	6,50E+00	2,17E+00	3,40E+01	4,64E+01	4,32E+00	

CHAPTER 5

Genome engineering of Stx1-and Stx2-converting bacteriophages unveils the virulence of the dairy isolate *Escherichia coli* O174:H2 strain UC4224

Genome engineering of Stx1-and Stx2-converting bacteriophages unveils the virulence of the dairy isolate *Escherichia coli* O174:H2 strain UC4224

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1. ABSTRACT

The past decade witnessed the emergence in Shiga toxin-producing *Escherichia coli* (STEC) infections linked to the consumption of unpasteurized milk and raw milk cheese. The virulence of STEC is primarily attributed to the presence of Shiga toxin genes (*stx1* and *stx2*) carried by Stx-converting bacteriophages, along with the intimin gene *eae*. Most of the available information pertains to the "Top 7" serotypes associated with STEC infections. The objectives of this study were to characterize and investigate the pathogenicity potential of *E. coli* UC4224, a STEC O174:H2 strain isolated from semi-hard raw milk cheese and to develop surrogate strains with reduced virulence for use in food-related studies. Complete genome sequence analysis of *E. coli* UC4224 unveiled the presence of a Stx1a bacteriophage, a Stx2a bacteriophage, the Locus of Adhesion and Autoaggregation (LAA) pathogenicity island, plasmid-encoded virulence genes, and other colonization facilitators. In the *Galleria mellonella* animal model, *E. coli* UC4224 demonstrated high pathogenicity potential with an LD_{50} of 6CFU/10µL. Upon engineering *E. coli* UC4224 to generate single and double mutant derivatives by inactivating *stx1a* and/or *stx2a* genes, the LD_{50} increased by approximately 1 Log-dose in the single mutants and 2 Log-doses in the double mutants. However, infectivity was not completely abolished, suggesting the involvement of other virulence factors contributing to the pathogenicity of STEC O174:H2. Considering the possibility of raw milk cheese serving as a reservoir for STEC, cheesemaking model was developed to evaluate the survival of UC4224 and the adequacy of the respective mutants as reduced-virulence surrogates. All tested strains exhibited the ability to survive the curd cooking step at 48°C and multiplied (3.4 Log CFU) in cheese within the subsequent 24h. These findings indicate that genomic engineering did not exert any unintended effect on the double *stx1-stx2* mutant behaviour, making it as a suitable less-virulent surrogate for conducting studies during food processing.

2. INTRODUCTION

Infections caused by Shiga toxin producing *Escherichia coli* (STEC) are responsible for outbreaks of serious diseases such as haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS), posing a serious public health concern (Pedersen et al., 2018). In 2020, 28 European countries reported 4,824 confirmed cases of infection with *E. coli* STEC and thus, recognized as the fourth most reported zoonosis (ECDC, 2022). Cattle have been recognized as an asymptomatic natural reservoir of STEC, representing a vehicle for human infection through direct contact or via foodstuffs (Zuppi et al., 2020). Recently, STEC outbreaks have been increasingly related to the consumption of dairy products; in Europe, two outbreaks in 2020 and one in 2021 as reported by EFSA-ECDC (EFSA and ECDC, 2021, 2022); in 2019, 20 paediatric cases of STEC O26:H11 infections were associated to the consumption of fresh raw milk cheese in France and other 21 cases were related to a milk pasteurisation malfunction at dairy farm level in UK (Jones et al., 2019; Jenkins et al., 2022). These outbreaks, as reported by the data collected

in the European Union One-Health (2022) report, demonstrate as raw milk cheese and other dairy products are frequently associated to the presence of STEC (2% of analysed dairy products (EFSA and ECDC, 2022)). Thus, in the absence of an effective pasteurisation process, the cheese production and ripening steps have proven to be insufficient to achieve the complete inactivation of these pathogenic *E. coli* microorganism (Bellio et al., 2018; Ioanna et al., 2018), as shown in studies that investigated the persistence of STEC in raw milk and its derivatives (Miszczycha et al., 2013; Peng et al., 2013; Ahmed and Samer, 2017). The current framework for identification of STEC includes the determination of serogroup, with correlation to their capacity to cause human illness. Serogroups O157, O145, O111, O103, and O26, considered the "top 5" STEC, have been identified as responsible for severe diseases and outbreaks (Franz et al., 2019; Koutsoumanis et al., 2020). Shiga toxins Stx1 and Stx2, encoded by genes *stx1* and *stx2* carried by lambdoid prophages, are considered the central driver of STEC virulence. Each of the Stx toxins are furtherly classified into subtypes and, particularly Stx2 subtypes a and c, seem to be corelated to the most severe forms of STEC diseases (Werber and Scheutz, 2019; Rodríguez-Rubio et al., 2021). The risk for a severe disease is generally associated with the concurrent presence of the *stx2* gene and the Locus of Enterocyte Effacement (LEE), which contains the *eae* gene, coding for the intimin protein responsible for adhesion (Franzin and Sircili, 2015). However, recently, non-O157 LEE-negative strains have been correlated with increasing number of infections in humans (Cundon et al., 2018; Krause et al., 2018; Colello et al., 2019; Cortimiglia et al., 2021). The LEE-negative STEC strains implicated in human disease harbour other virulence factors (VFs) involved in other adherence processes carried by plasmids, non-Stx prophages or unique pathogenicity islands (PAIs). Montero et al. (Montero et al., 2017), described the PAI Locus of Adhesion and Autoaggregation (LAA), a 86 kb region divided in four modules containing the *hes* gene coding for and haemagglutinin (Montero et al., 2017). A recent study by Cortimiglia et al. (Cortimiglia et al., 2021) detected this virulence locus in STEC O174 strains harboring both Stx1- and Stx2- bacteriophages isolated from Italian semi-hard raw milk cheese. Moreover, *E. coli* O174 strains are frequently detected as being among the top 10 STEC serotypes from animal, food and humans (EFSA and ECDC, 2022). Although the risk that STEC poses for consumers of dairy products is high, few studies have addressed the growth, survival and inactivation kinetics of Shiga toxin producing *E. coli* during the cheese processing and ripening (Schlesser et al., 2006; Miszczycha et al., 2016; Centorotola et al., 2021). One of the major limitations in the development of challenge studies in food, is the high pathogenicity of STEC strains that hamper their use in pilot plants outside the confined conditions of biosafety laboratories. STEC mutants with the toxin genes inactivated were developed to assess the role of Stx virulence (Kim et al., 2010; Xue et al., 2011) but not specifically used as surrogate for toxigenic strains to appraise the growth and persistence in food models. The objective of our study is to perform a comprehensive genomic characterization of the virulence profile of *E. coli* UC4224, a STEC strain isolated from semi-hard raw milk cheese, utilizing a WGS-based approach. Our research also aims to investigate the impact of *stx1* and *stx2* genes on pathogenicity *in vivo* using the *Galleria mellonella* model by individually and collectively inactivating them via genome engineering.

Moreover, we assessed the survival of the parental strain and the suitability of the three mutants as attenuated surrogates under acid stresses and in cheesemaking conditions.

3. MATERIALS AND METHODS

3.1 Bacterial strains, plasmids, and media

STEC strain UC4224, isolated from semi-hard raw milk cheese, and respective mutant strains were grown in Luria-Bertani (LB) broth (Sigma-Aldrich) and supplemented with appropriate antibiotics when needed. The antibiotics used were chloramphenicol (Cm) (Sigma- Aldrich) (3.125–25 μg/mL), kanamycin (Kan) (Sigma-Aldrich) (12.5–25 μg/mL), and ampicillin (Amp) (Sigma-Aldrich) (100 μg/mL). Strains and plasmids used in this study are listed in Table 1 whereas oligonucleotides are listed in Supplementary Table S1. The *E. coli* strain DH5α, grown in LB broth supplemented with Amp, was used for the propagation and purification of plasmids.

Table 1 Bacterial strains and plasmids used in this study.

Strain	Relevant genotype, phenotype	Reference/Source
E. coli		
UC4224	STEC parental strain	This study
UC4175	UC4224(pSIM6), Amp^R (Ts)	This study
UC4176	UC4224⊿*stx1::kan*, Kan^R	This study
UC4177	UC4224⊿*stx2::cat*, Cm^R	This study
UC4178	UC4224⊿*stx1::kan* ⊿*stx2::cat*, Kan^R Cm^R	This study
Plasmids		
pSIM6	Plasmid expressing Lambda red recombination genes below the control of CI857 repressor, Amp^R (Ts)	Datta et al. (2006)
pKD3	Template plasmid for the amplification of FRT-cat-FRT amplicon, Amp^R Cm^R	Datsenko and Wanner (2000)
pRL128	Template plasmid for the amplification of FRT-kan-FRT amplicon, Amp^R Kan^R	Gueguen and Cascales (2013)

Amp, ampicillin; Kan, kanamycin; Cm, chloramphenicol; superscripts "R" and "S" represent resistance and sensitivity, respectively.

3.2 Whole genome sequencing and data submission

Genomic DNA of UC4224 and UC4178 (UC4224⊿*stx1::kan*⊿*stx2::cat*) was extracted from 1 mL of an overnight culture by E.Z.N.A. ® Bacterial DNA Kit (Omega Bio-tek), following the manufacturer's instructions. After, Qubit 2.0 Fluorometer (Thermo FisheR Scientific) was utilized to quantify the DNA concentration and then loaded on agarose gel (0.8%) to confirm the DNA integrity. Genomic DNA of UC4224 and UC4178 were sequenced using Illumina Miseq platform with 250 paired-end run after Nextera XT paired-end library preparation (Illumina). Additionally, long-read sequencing was carried out for UC4224 only and performed with PacBio Sequel II SMRT sequencing. Sequence trimming was completed with trimgalore! (GitHub – FelixKrueger/TrimGalore) (Krueger, 2016). After, hybrid assembly was executed using Unicycler (Wick et al., 2017). Then, contigs of both parental and mutant strains, were annotated with Prokka with a de fault e-value cut-off (version 1.13.3) (Seemann, 2014). Genome assemblies were deposited on NCBI under Genbank assembly accession No. GCA_025369975.1 for UC4224 and GCA_025290845.1 for UC4178.

3.3 Bioinformatic analyses

A total of 99 strains genomes (including UC4224) were retrieved from NCBI for phylogenomic analyses, including 40 from cheese, 5 from dairy milk and 54 from non-specified dairy products (Supplementary Table S2). Bioinformatic analyses comprising the calculation of the pangenome and the construction of the phylogenetic tree with bootstrapping of 1,000, of all genomes were performed as previously described by Belloso Daza et al. (2021). The screening for virulence factors, antimicrobial resistance genes was executed according to another study (Cortimiglia et al., 2021). Finally, the analysis of mobile genetic elements like plasmids and prophages was carried out following the pipeline of Belloso Daza et al. (2022).

3.4 Construction the amplimer with short (50bp) and long (~280bp) homology sequences

PCR reactions were performed using Phusion Flash High-Fidelity PCR Master Mix (ThermoFisher Scientific) as provided by the manufacturer. The two plasmids pKD3 (Datsenko and Wanner, 2000) and pRL128 (Gueguen and Cascales, 2013) were used for amplifying the resistance cassettes using primers (Supplementary Table S1) constructed as described by Egan et al. (2016). The detection of each amplicon was verified by gel electrophoresis (ThermoFisher Scientific), then the product was excised from the gel and purified using the Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up (Macherey- Nagel). The PCR product was concentrated using Zymo Research's DNA Clean & Concentrator Kit™-25 (D4005) in a final volume of 25 µL of molecular-grade water.

Homology regions of the *stx2* gene, located at the two ends of the *cat* cassette, have been increased as previously described by Serra- Moreno et al. (2006). The new PCR products were constructed using the overlapping regions within three different dsPCR fragments: the *cat* cassette and the other two that present homology with both *stx2* and antibiotic resistance cassette using the primers reported in Supplementary Table S1. The amplimers obtained were *stx*2 Forward/ Cm-F *stx2* (270 bp) and *stx*2 Reverse/Cm-R *stx2* (280 bp). The three amplimers obtained were annealed at their overlapping region. The two external primers *stx*2 Forward and *stx*2 Reverse were used to overlap the three fragments. The fusion product was amplified using the same primer pair *stx*2 Forward/Reverse, subsequently purified. The fusion product obtained is ⊿*stx2::cat* PCR amplicon with long homologous arms.

3.5 Transformation of *Escherichia coli* STEC UC4224 with plasmids pSIM6 and preparation of electrocompetent cells for recombineering

The pSIM6 plasmid was propagated in *E. coli* strain DH5α and extracted using ZymoPURE Plasmid Miniprep Kit (Zymo Research) following the manufacturer instructions. Then it was transformed in UC4224 after making it electrocompetent (BIORAD, 1900) *E. coli*. The transformant of UC4224 with the pSIM6 was named UC4175. The UC4175 overnight culture was then diluted to 100-fold in LB with Amp (100 µg/mL) and grown to an OD_{600} of 0.8. The culture was then thermally shocked at 42°C at 250 rpm for 45 min to induce the lambda red genes expression by pSIM6, as described previously (Egan et al., 2016). After the induction, UC4175 was made electrocompetent as described above. Ninety µl of chilled electrocompetent UC4175 cells were added to 100 ng of ⊿*stx2::cat* or ⊿*stx1::kan* PCR amplicons, including negative controls without PCR products. The mix was held on ice for 1 min, then, electroporation was performed at a voltage of 2.5 kV. Electroporants were immediately recovered in 1 mL of S.O.C medium and grown at 37°C at 225 rpm overnight,. After, the cultures were spread on LB supplemented with Cm (6.5–25 µg/mL) or Kan (15–30 µg/mL) and examined to determine Cm^R and Kan^R recombinants. Recombinants were observed after 1 to 2 days of incubation at 37°C. The resulting mutants are UC4176 (UC4224⊿*stx1::kan*, Kan^R) and UC4177 (UC4224⊿*stx2::cat*, Cm^R) (Table 1).

Subsequently, UC4176 was induced and made eletrocompetent, as described above. The induced eletrocompetent cells were eletroporated with 100 ng of ⊿*stx2::cat* PCR amplicons with long homologous arms. The electroporation conditions used were the same as those described previously. After 16 h of incubation, recovered cultures were cultured onto LB plates with Cm (6.5–25 µg/mL) and Kan (15–30 µg/mL) and examined to determine Cm^R/Kan^R recombinants. The resulting double mutant is UC4178 (UC4224⊿*stx1::kan* ⊿*stx2::cat, Kan*^{R} *Cm*^{R}) (Table 1).

3.6 Curing of pSIM6 and replacement confirmation

Once the recombinant UC4178 had been found, 10 µL of an overnight culture was spread on LB agar with added Cm (6.25 µg/mL) and Kan (22.5 µg/mL) and incubated overnight at 42°C. A few colonies were then taken and streaked onto both LB agar supplemented with respective antibiotics and incubated overnight at 30°C. The correct replacement was confirmed by locus specific PCR and Sanger sequencing. Briefly, the *stx1* and *stx2* genes were amplified with the external primer listed in Supplementary Table S1 (Paton et al., 1993, 1995; Ruessmann et al., 1994; Muniesa et al., 2003), visualized on 1.2% agarose gel by Sybr-Safe staining (Thermofisher) and purified using ReliaPrep DNA clean-up and concentration system (Promega) according to the protocol provided by the manufacturer. The purified DNA was sequenced by commercial facility (Eurofins Genomics, Italy) using Sanger technology. Additionally, the replacement was confirmed through WGS, performed as reported above. The genome assembly is deposited in Genbank with accession number GCA_025290845.1.

3.7 Pathogenicity assessment in *Galleria mellonella*

The *in vivo* analysis using larvae of the greater wax moth, *G. mellonella*, was performed as previously described by Morgan et al. (2014). Briefly, bacterial overnight cultures were pelleted and washed twice in Phosphate Buffer Solution (PBS) (0.1 M) and resuspended in 10 mL of PBS. The larvae were selected to be 15–25 mm long, cream- coloured with minimal spotting and no grey marks. Three independent biological replicates of ten larvae of 200 to 250 mg each, were infected with 10 µL of serial dilution (10^1–10^7 CFU/10 µL) of each different strains, by injection with a 26-gauge Hamilton syringe. Larvae were then incubated at 37° C in the dark and the dose resulting in 50% of kills (LD_{50}) was calculated after 24 h. The survival rate was monitored for an additional 48 h. The strains used for this assay were UC4224, UC4176, UC4177, UC4178 and *E. coli* BL21 and PBS only as a negative control. An additional control composed by three groups ($n = 10$) without manipulation, was added. Kaplan–Meier survival curves were constructed to evaluate the probability of survival of the different strains at different injection doses using GraphPad Prism (Survival curve 8.4.3 (686)). Logrank tests were applied to detect any significant differences in survival rates between strains ($p < 0.05$). Microbial count of bacteria was realized to verify the inoculated doses onto LB agar supplemented with Kan and Cm, when needed. The LD_{50} values were calculated using Probit Analysis, following the methodology of Finney (1971) in Excel 2010 with a 95% confidence limit (Mekapogu, 2021).

3.8 Cheesemaking model and tolerance to lactic acid

We assessed the survival capacity of UC4224 and the suitability of UC4176, UC4177 and UC4178 as attenuated surrogates under acid stresses and in cheesemaking process. The cheesemaking process was carried out according to the traditional production method from raw milk. Briefly, 200 mL of raw milk was aliquoted in five different 500 mL flasks and pre-warmed at 30°C. Once the desired temperature was reached, each flask was inoculated with a mix of three different starter cultures: *Streptococcus thermophilus*,

Lactococcus lactis and *Lactobacillus delbrueckii* subsp. *lactis* at a cell numbers of 10^7 CFU/m each, and 0.2 mL of rennet per litre of milk. Subsequently, four flasks prepared as described above were individually inoculated with 200 µL of an overnight culture of UC4224 and the three mutants (inocula-t0); the remaining flask, without *E. coli* inoculum, was used as a negative control. The five samples were heat-treated at 34°C for 40 min (t1); then, the temperature was increased at 48°C for 40 min (t2) and finally the curds were packaged, pressurised and drained; thus, stored at room temperature (20°C) for 24 h (t3). Plate counts were carried out in triplicate at times t0, t1, t2 and t3 using Violet Red Bile Glucose Agar (Oxoid), supplemented with Kan 50 µg/mL and Cm 50 µg/mL when required, and incubated at 37°C for 24 h. Lactic acid tolerance was tested as previously described by Liu et al. (2020) with slight variations. Shortly, the overnight culture of the parental strain and the three mutants were serially diluted and 10 µL of each dilution were spotted on LB agar, modified with L-lactic acid (Carlo Erba) to a pH of 4, 4.5, 5, 5.5, 6 and 6.5, and incubated at 37°C for 24 h. All results were statistically analysed using the Tukey's pairwise test, via the *Past4.06b* software, with $a = 0.05$.

4. RESULTS

4.1 Genome sequencing and characterization of UC4224

In this study, *Escherichia coli* STEC strain UC4224, isolated from semi-hard raw milk cheese, was investigated for its resistome/ virulome/mobilome. The first step toward identifying the nature of this strain was to perform WGS following a long-and short-read approach. After sequencing and quality check, UC4224 was assembled into 3 molecules, one chromosome of 5,047,333 bp and two plasmids, pUC4224_1 (111,840 bp) and pUC4224_2 (6,883 bp). UC4224 was identified as ST 661, serotype O174:H2 and Clermont phylogroup B1.

4.2 Phylogenomics and distribution of dairy associated STEC

Phylogenomic analysis was performed to understand the relationship of *E. coli* UC4224 with a selection of 95 *E. coli* genomes, retrieved from NCBI, isolated from dairy-associated sources: milk, cheese and dairy products. The pangenome analysis resulted in a total of 2,175 (8.7%) core genes, 2,652 (8.3%) shell genes and 25,653 (83%) cloud or accessory genes. This outcome is in line with the concept of the open pangenome of *E. coli*, decreasing core genomes and increasing accessory genomes that support the adaptability of *E. coli* from different ecological niches and the diversity of strains with pathogenicity for animals and human (Tantoso et al., 2022). Furthermore, core-genome derived data was then used to construct a maximum likelihood phylogenetic tree. As observed in Figure 1, no clear cluster patterns regarding serogroup or Stx type are noticeable. When observing the relative abundance of the serogroups we found the most frequent serogroups were O157 (17%), O6 (13.8%), O26 (8.5%) and O174 (7.4%). Other recurring serogroups were O103 (6.4%), O5 (5.3%) O8 (4.3%), O110 (3.2%), O116 (3.2%), O145

(4.3%). Out of 94, only 6 isolates (6.4%) were not assigned to any serogroup. A strict relationship between the Stx-bacteriophages and serogroups results from the phylogenomic analysis, as reported in other studies (Zhang et al., 2022). In particular, O174 strains harbour both Stx1- and Stx2-converting phages and O6 and O26 dairy isolates carry Stx1. Higher variability was observed in O157 which may contain either Stx2 – or both Stx1- and Stx2-phages.

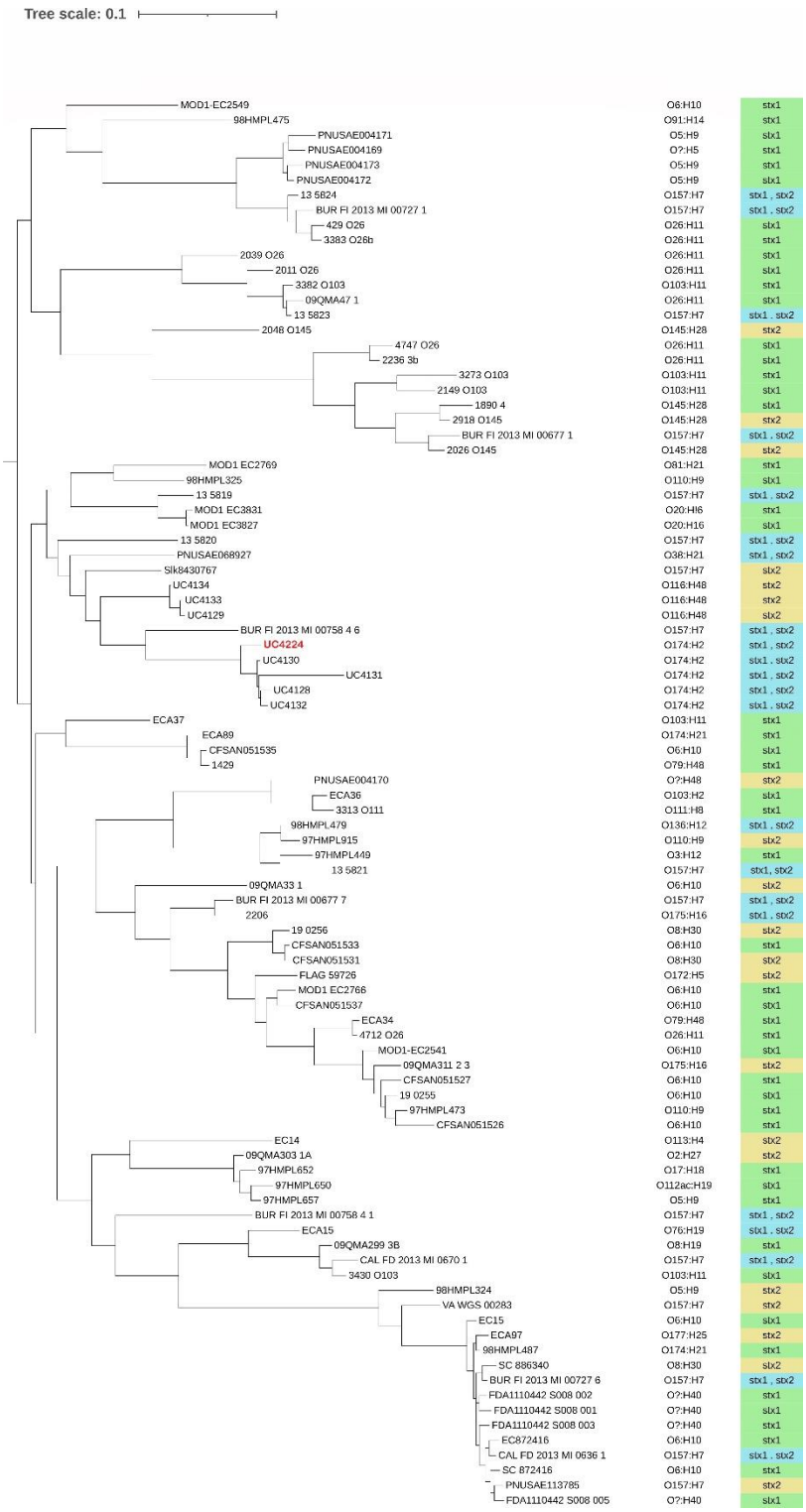

Figure 2 *Maximum likelihood phylogenetic tree using core genes alignment of 99 dairy-associated strains retrieved from NCBI. UC4224 is depicted in red. The serogroup of each isolate was determined using a bioinformatic tool and it is presented on the right side of the three. The type of Shiga toxin is depicted in green for Stx1, yellow for Stx2 and blue for Stx1 and Stx2. The variability of the presence of Stx-type is correlated to the serogroup. No clear patterns in serogroup or Stx-type distribution is observed. The selected strains had different isolation sources: milk, cheese and dairy products; their distribution can be found in Supplementary Table S2.*

4.3 Stx-converting phages and other prophages

Genome scrutiny of UC4224 revealed the presence of *stx1a* and *stx2a* carried by two separate prophages. We comprehensively explored the two Stx-phages and their respective flanking regions by identifying the attachment sites, structural and regulatory regions. Stx-phages are double-stranded DNA-phages with a functional genetic organization comparable lambda phage, as it is the case of UC4224 phages. Stx1-phage of size 62.2 kb (Figure 2B), was found in positions 669,258–731,903 bp, with the highest homology score to Enterobacteria phage D3 (NC_042057). Stx2-phage, of size 77 kb (Figure 2C), found in position 4,824,523–4,901,603 bp with highest similarity to phage D3, as well. Both phages were found to be unique with the highest BLAST nucleotide identity of 92 and 88% with other Stx-phages for Stx1-phage and Stx2-phage, respectively.

The *stx* genes loci of both phages, were composed by the two *stx* genes coding for subunits a and b, the antitermination protein Q, responsible for late-phase transcription regulator and lysis protein S. Downstream from the Stx-region of both phages, lysis, terminase and structural proteins coding for capsid, tail and tail fibers were observed. Regulation and recombination genes were found adjacent to the toxin and structural genes regions. Upstream from the Stx-region, recombination coding sequences were found (Figures 2B,C). Specifically, in Stx1-phage, gene *nu1* was found, coding for a typical protein for DNA packaging in Stx-converting phages (Figure 2B). Stx2-phage carries *perC,* a Type 3 Secretion System (T3SS) expression regulator related to the expression of LEE-encoded virulence factors in STEC (Carter et al., 2021).

Moreover, four additional prophages were predicted chromosomally, namely a 25.4 kb phage with highest homology to Enterobacteria phage YYZ_2008 (Acc. No. NC_011356), two phages of 39.1 kb and 44.7 kb similar to Enterobacteria phage lambda (Acc. No. NC_001416) and a 46.9 kb closest to *Klebsiella* phage 4 LV-2017 (Acc. No. NC_047818). The 44.7 kb phage carried gene *ompT* (outer membrane protein and serum resistance lipoprotein *bor* was found flaking the lysis and terminase regions of this phage. Downstream from the structural region and adjacent to the tail fibers coding genes, *lom* (outermebrane protein) was found (data not shown).

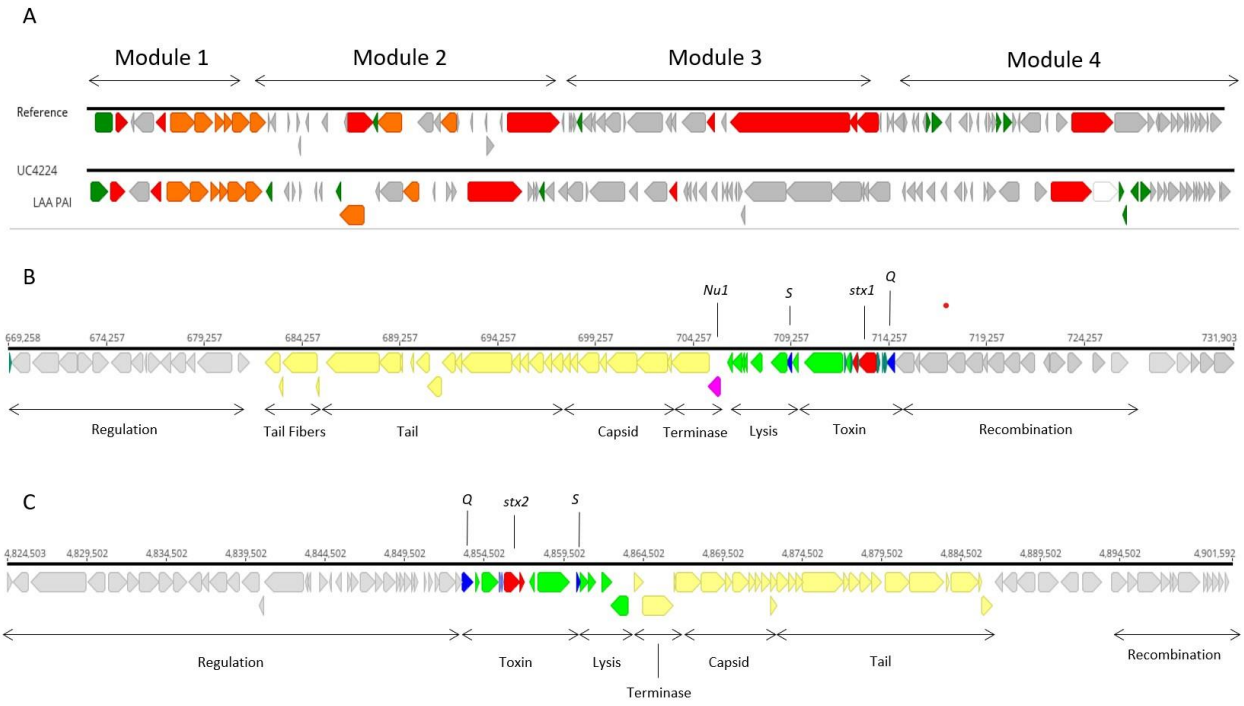

Figure 3 *Stx1- and Stx2-converting phages and LAA PAI in UC4224.* ***(A)*** *sequence alignment of LAA PAI reference sequence from E. coli B2F1 (Genbank accession AFDQ01000026) and UC4224.* ***(B)*** *genomic annotation of Stx1-phage including stx1a toxin subunits (red), antiterminator protein Q and holin S (blue), Nu1 protein (magenta), lysis and toxin operon CDS (green), structural proteins for capsid and tail (yellow) and, regulation, recombination and other CDS (grey). Stx 1 attachment site attL was found in position 669,258–669,271bp (13bp, TGCCGGATGCGGCG) and attR in position 731,916– 731,903 (13bp, TGCCGGATGCGGCG).* ***(C)*** *genomic annotation of Stx2-phage including stx2a toxin subunits (red), antiterminator protein Q and holin S (blue), lysis and toxin operon CDS (green), structural proteins for capsid and tail (yellow) and, regulation, recombination and other CDS (grey). Stx2- phage presented attL in position 4,830,701–4,830,714bp (13bp, TGGATGATTTTTCA) and attR in 4,901,592–4,901,603bp (11bp, TTATGAAAAACG).*

4.4 Virulence factors and LAA pathogenicity island

Although *stx* genes are considered the main drivers of virulence, *E. coli* STEC strains have developed pathogenicity islands (PAI) carrying genes for adhesion and colonization and attachment that facilitate the expression of virulence within the host. *E. coli* UC4224. does not harbour the Locus of Enterocyte Effacement (LEE) PAI, a 35.6 kb region containing genes responsible for causing attaching and effacing lesions, characteristic of *E. coli* O157: H7 (Franzin and Sircili, 2015). Differently, the WGS scrutiny revealed the presence of a region showing 60.8% nucleotide similarity with the Locus of Adhesion and Autoaggregation (LAA) PAI (Genbank Acc No. AFDQ01000026) (Figure 2C), a genetic locus described by Montero et al. (Montero et al., 2017). As shown in Figure 2A, module 1 carries the gene *hes*, involved in self-aggregation and adhesion (Vélez et al., 2020a). Module 2 habours the *lesP* gene, which encodes a variant of an enterobacterial self-transporting serine protease (SPATE) (Montero et al., 2019). Module 3 has the *pagC,* an outer membrane protein important in serum resistance in *Salmonella enterica* (Hasson et al., 2022).

Finally, the *agp43* gene is found in module 4, which is related to the capacity for self-aggregation and accumulation of cells, which promotes biofilm formation (Montero et al., 2017).

Other two PAIs have been described to appear in LEE-negative STEC strains, specifically: the Locus of Proteolysis Activity (LPA) (Hauser et al., 2013) and the Subtilase-Encoding Pathogenicity Island (SE-PAI) (Bondì et al., 2017) were not found in *E. coli* UC4224. Other chromosomally located genes encoding for adhesins, T3SS effectors and potential virulence factors were identified, including *hra* (heat-resistant agglutinin) and long polar fimbriae (*lpfA*), an important factor for STEC intestinal colonization and adhesion (Supplementary Table S3) (Toma et al., 2006; Vélez et al., 2022). The IncF-type conjugative plasmid pUC4224_1 (111 kb), carries a large Integrative Conjugative Element (ICE) in position 25,634–106,706 bp (81,073 bp). This ICE presents an origin of transfer (*oriT*), Type 4 Secretion System (T4SS) proteins tra and trb and Type IV coupling protein (T4CP) in ORF 48 (795 aa). Moreover, pUC4224_1 carried several potential virulence factors (Supplementary Table S3), among them adherence protein *iha,* enterohemolysin operon *ehxABCD*. Next, gene *espP* was also found, these genes are homologues members of Serine Protease Autotransporters of Enterobactericeae (SPATE) family. The *traT* gene, a plasmid-located determinant encoding for an outer membrane protein that inhibits the membrane- attack complex present in the serum of the host (Miajlovic and Smith, 2014) and *saa* (STEC autoagglutinating adhesin) genes (Cundon et al., 2018) were found as potentially involved in virulence. This strain harbours colicin coding genes *cia* and *celb*, considered as a putative virulence factors as they facilitate colonization (Micenková et al., 2017). Furthermore, several stress response systems regulators were found in UC4224, gene list with corresponding gene function are listed in Supplementary Table S3.

4.5 Construction of *stx1* and *stx2* null mutants

To investigate the role of phage encoded *stx* genes from newly identified Stx-phages from a non-O157 strain isolated from semi-hard raw milk cheese. Therefore, we constructed *stx1*- and *stx2*-knock-out strains by inserting antimicrobial cassettes by using the lambda red recombination system expressed by the low copy plasmid pSIM6, as shown in Figure 3. After the deletion of *stx* genes, PCR experiments and Sanger sequencing confirmed the substitution of the *stx1* region with the KanR (*aph(3')-IIa*) (UC4176 and UC4178) and the *stx2a* with the CmR (*catA1*) (UC4177 and UC4178), in all the three obtained mutants as shown in Figure 3. Moreover, the WGS analyses of UC4178 confirmed the double substitution of the *stx* genes and the absence of the pSIM6 plasmid and, no other differences were observed when compared with the parental strain UC4224.

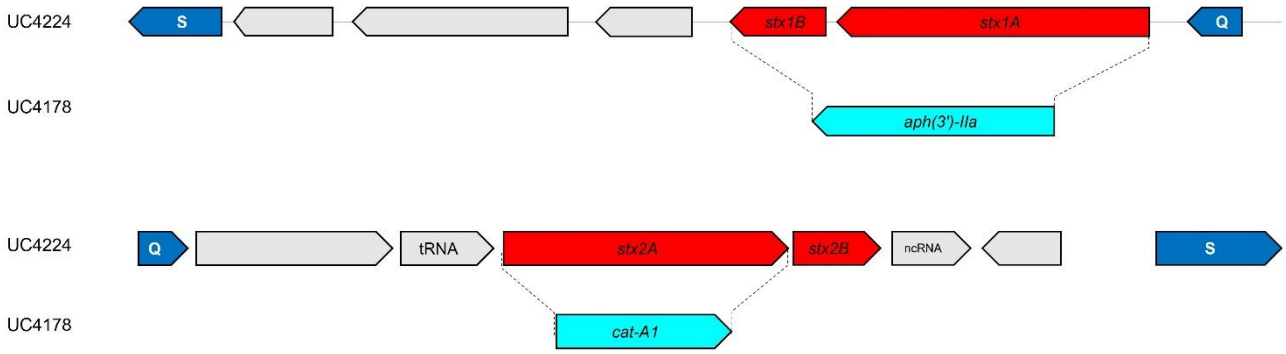

Figure 4 *Schematic representation of stx1 and stx2 replacement in UC4224. Genes are represented by arrows. In cyan the antimicrobial resistance cassettes. In red the two subunits, respectively, of stx1 and stx2 genes. In the double mutant UC4178, the replacement event occurred via homologous recombination between stx1 subunits a/b and the kanamycin resistance cassette and between stx2a and the chloramphenicol resistance cassette. The dimension of the amplified stx1 and stx2 genes, with the external primers, in the parental strain UC4224 have a size, respectively, of 1,281bp and 1,241bp. Instead, the size of the same region, amplified with the same primers, in strain UC4178 are, respectively, of 1,403bp and 1,568bp; thus, confirming the correct gene substitution.*

4.6 Lethality in the *Galleria mellonella* model of UC4224 and its derivative mutants is correlated with carriage of *stx* genes

In this study we tested the virulence of STEC UC4224 and STEC- negative mutants UC4176, UC4177 and UC4178. To determine the mortality rates, the *G. mellonella* larvae were injected with a range of 10^1 to 10^7 CFU/10 µL of the mutant strains in comparison with UC4224 and *E. coli* BL21 as negative control. Larvae injected with negative controls showed no mortality. The Kaplan–Meier survival analysis (Figure 4) was based on the four lowest doses, as at 1.8×10^4 CFU/10 µL or higher, and the observed mortality rate was 100% for all the tested strains. The parental strain UC4224, which harbours the two intact *stx* operons, has a LD_{50} of 6.0 CFU/10 µL (Supplementary Table S5.2). When the two single mutants UC4176 and UC4177 were tested, the observed mortality rates were significantly lower than UC4224 ($p < 0.05$) for the three lowest doses injected, with a LD_{50} of 81.7 CFU/10 µL and 50.5 CFU/10 µL respectively, but not significantly different between them (Supplementary Table S5.1). The lethality rate of UC4178 strain, with a LD_{50} of 582.7 CFU/10 µL, was significantly lower than the parental strains and the two single mutants for the four tested doses. *In vivo* trials with *G. mellonella* indicated an improved survival rates in larvae samples treated with the three mutants compared to those treated with the parental strain, with particular attention to UC4178 in which the deletion of the *stx1* and *stx2* genes allowed a considerable reduction in pathogenicity.

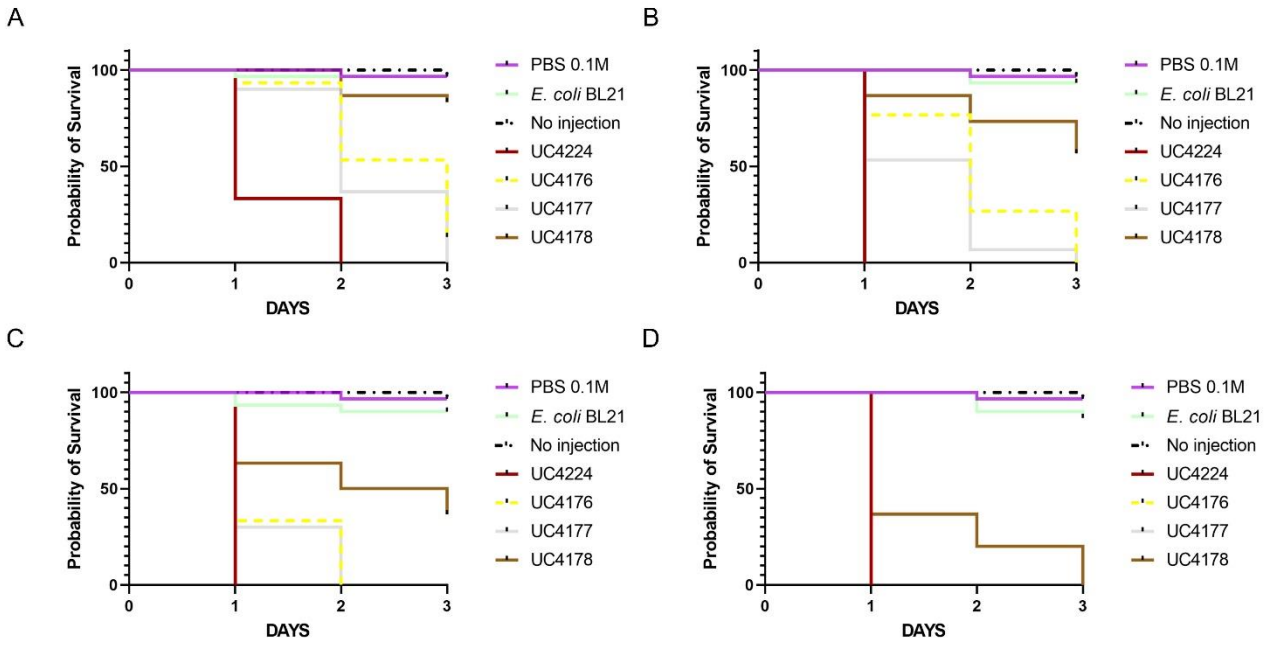

Figure 5 *Kaplan–Meier survival curves of the experiments with G. mellonella larvae inoculated with tested strains at different injection doses* ***(A)*** *9CFU/10µL,* ***(B)*** *1.8×10¹ CFU/10µL,* ***(C)*** *1.8×10² CFU/10µL, and* ***(D)*** *1.8×10³ CFU/10µL. Each group contained 30 larvae separated in three groups of 10 larvae. E. coli.*

4.7 Survival and growth dynamics of UC4224 and derivate mutants during food processing

A cheesemaking model, mimicking the first step of raw milk cheese production, was developed to evaluate the survival of UC4224 and the adequacy of the respective mutants as reduced-virulence surrogates during the cheesemaking process. The results of the bacterial counts, expressed as the average of three experiments, are shown in Figure 5. The four considered strains showed the same inactivation and growth dynamics in all the analysed steps of the food processing model, without statistically significant differences. In the first 40 min at 34°C, corresponding to the renneting step, no growth was observed. The thermal treatment at 48°C for 40 min, which represents the typical step of semi-hard raw milk cheese, resulted in a reduction greater than a 2 Log CFU/g for all four strains. In the subsequent step, when the curd was separated from whey and maintained at 20°C for 24 h, growth was observed reaching values of 1.3 Log and 3.4 Log to the thermal treatment at 48°C step. *E. coli* UC4224 and its Stx-phage-inactivated strains derivative were tested for their resistance to pH values typical of dairy products, obtained by adding lactic acid to growth medium. At pH 4, no growth was detected for any of the four strains at any cell density tested, at pH 4.5 growth was observed only with an inoculum concentration higher than 7 Log CFU/ml, while at pH of 5 was not growth limitation was seen (Supplementary Table S4).

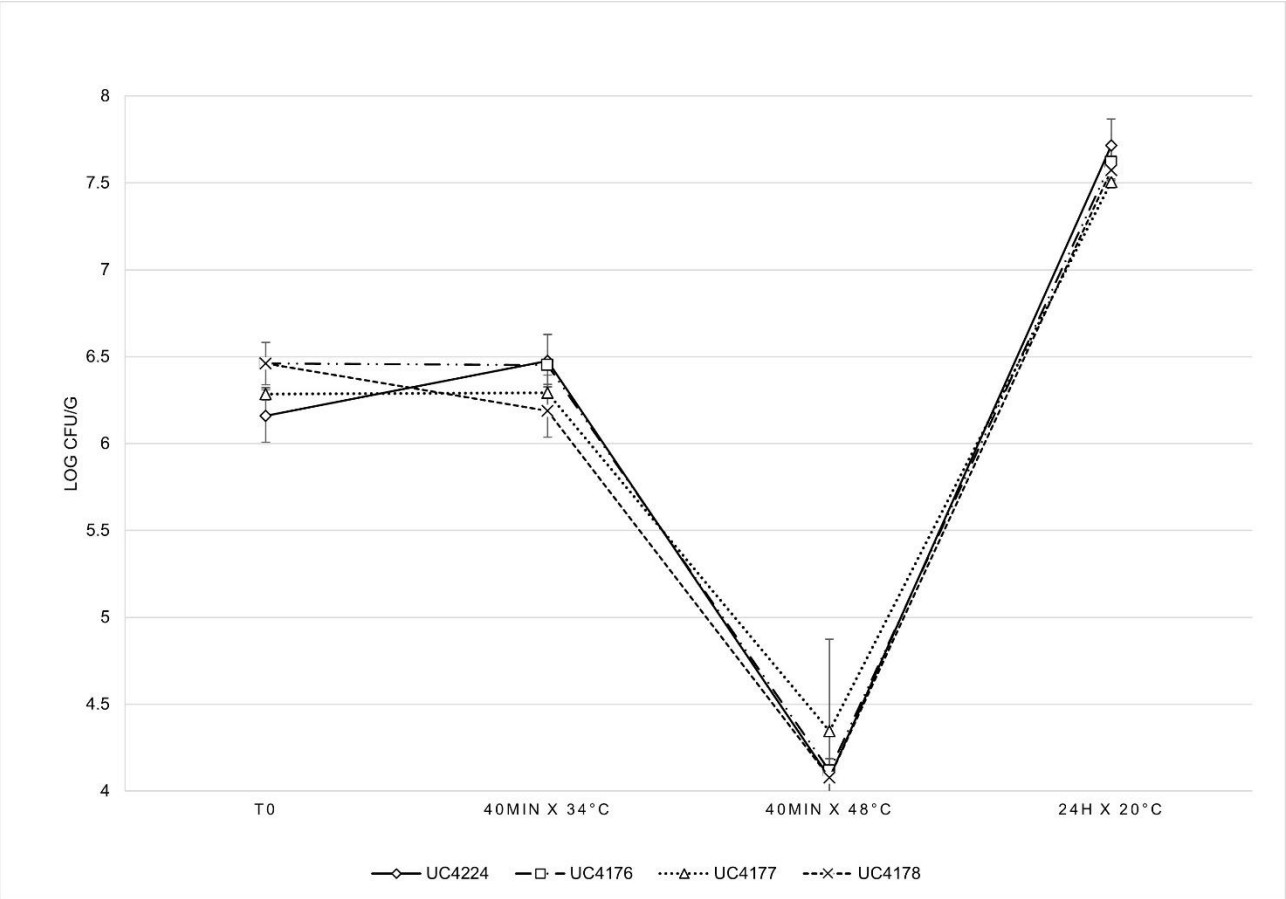

Figure 6 *E. coli counts in cheese making, at different analysis times, for the four independent experimental tests expressed as the average of three independent experiments. Error bars indicate standard deviation.*

5. CONCLUSION

Recently, Shiga-toxin producing *Escherichia coli* (STEC) infections have been associated with the consumption of raw milk and derivatives thereof. In this study, STEC strain UC4224 was isolated from semi-hard raw milk cheese and was subjected WGS to investigate its virulence profile. Bioinformatic analyses using genome-derived data, allowed the classification of UC4224 as ST 661, serotype O174:H2 and, carrying two new and separate Stx1 and Stx2- converting phages with the typical Stx-converting phage structure (Figure 2). Many *stx*-carrying strains harbour LEE PAI but this strain was determined LEE-negative. LEE-negative strains have developed further mechanisms that facilitate infection. Commonly, LEE-negative STEC strains carry other adhesion and colonization-contributing factors like *iha* (IrgA homologue adhesin), *saa* (STEC autoagglutinating adhesin), and *lpfA* (long polar fimbria), that compensate for the absence of LEE, as it is the case of UC4224 (Lorenz et al., 2016). UC4224 harbours also LAA PAI (86 kb) in the chromosome and carries virulence factors throughout its 4 modules as previously described (Montero et al., 2017). A 44.7 kb non-*stx* prophage was found in the chromosome, with the gene *ompT*, a gene coding for a membrane protease highly associated with adhesion and pathogenicity in urinary tract infections (He et al., 2015); *lom* and *bor* genes were also found, which are

involved in T3SS expression that confer serum resistance and enhance adhesion (Rodríguez-Rubio et al., 2021). It has been demonstrated that other non-*stx* prophages have a direct impact on the STEC pathogenicity and pangenome, but the direct impact on UC4224 virulence is still to be determined (Rodríguez-Rubio et al., 2021). Other plasmid encoded virulence genes were found in pUC4224_1 (111 kb), including enterohemolysin gene *ehxA*, demonstrated to contribute to virulence in STEC (Lorenz et al., 2016; Hua et al., 2021); SPATE family gene (*espP*) and other adhesion (*traT* and *saa*) genes.

Insights into the virulence profile of UC4224 led to the construction of single and double *stx1* and *stx2* knock-out mutants to study its pathogenicity potential *in vivo* and evaluate their adequacy as surrogates with reduced pathogenicity during cheesemaking. With a genome engineering approach we generated three mutant strains in which genes *stx1* and *stx2* were substituted with antibiotic resistance cassettes to create UC4176(⊿*stx1::kan*, KanR), UC4177(⊿*stx2::cat*, CmR) and UC4178(⊿*stx1::kan* ⊿*stx2::cat*, KanR CmR), as confirmed by Sanger sequencing. Previous studies have deleted both *stx1* and *stx2* from STEC O157:H7 (Yokoyama et al., 2001; Ma et al., 2011), without evaluating the pathogenicity *in vivo*. We focused the first part of this study to assess the role of Shiga toxins in UC4224 and respective mutants *in vivo*. Our results showed that, when considering the deletion of either or both the *stx* genes, all three mutants presented differences in the lethality against *G. mellonella* larvae when compared to the parental strain. In particular, we observed that the double mutant UC4178 ⊿*stx1* ⊿*stx2* showed highly reduced virulence with an increased LD_{50} of 2 Log dose when compared to UC4224 which shows a LD_{50} of 6 CFU/10 µL. Our study indicates that the presence of both *stx1* and *stx2* genes have a combined effect on the pathogenicity of STEC, in fact the single mutants UC4176 ⊿*stx1* and UC4177 ⊿*stx2* showed a lower virulence (1 Log increase of median lethal dose) than UC4224. No differences between strains producing Stx1or Stx2 toxins were detected in the *G. mellonella* model, differently from what was observed in other animal models (Xue et al., 2011). In line with our results, a previous study has shown that non-pathogenic *E. coli* strains are non-lethal to *G. mellonella* with inoculations of up to 107 CFU/ larvae (Zuppi et al., 2020). Our results highlight that the deletion of either or both the *stx* genes does not completely suppress UC4224 virulence, leading to suppose the involvement of LAA PAI, plasmid- encoded VFs, non-*stx* prophage encoded VFs, non-LEE T3SS effectors and other colonization contributing factors in delivering pathogenicity to the host (Cundon et al., 2018; da Campos et al., 2019; Vélez et al., 2020b; Cortimiglia et al., 2021; Sánchez et al., 2021). Indeed, previously, another study observed that the deletion of *stx* genes in the presence of other virulence factors reduces the pathogenicity. In this work, Habets et al. (2022) showed that non-STEC EPEC O80:H26*E. coli* strains which correctly transduced with the Stx2d-phage, increased lethality in *G. mellonella* larvae, proving that the Stx2-phage confers partial virulence to a strain harboring other virulence factors (Habets et al., 2022).

After establishing the mutants as suitable substitutes with diminished virulence, the second part of our study focused on the evaluation of the phenotypic differences between the mutants and the parental strain. The three mutants and the parental strain were submitted to a pilot scale raw milk cheese production to assess their survival in the cheese matrix, which is typically subjected to different stressing conditions like

temperature, pH, a_w and redox potential changes. The possibility to use less virulent strains to study how it reacts within cheese manufacturing is important in challenge tests to avoid using hazardous pathogens. The intrinsic attributes of cheese, related to the different production and ripening processes, should act as a barrier to bacterial growth. Along with this, the intrinsic microbiota of raw milk together with the starter cultures are expected to outcompete pathogens by lowering the pH (Baylis, 2009). Nevertheless, raw milk cheeses of different varieties (soft and semi-hard) have been described as sources of contamination or outbreaks of STEC, since they do not undergo pasteurisation and the production process is not effective in counteracting the proliferation of these bacteria (Schlesser et al., 2006; Caro and García-Armesto, 2007; Miszczycha et al., 2013, 2016; Peng et al., 2013; Ahmed and Samer, 2017; Celikl et al., 2021). However, STEC have been isolated from pasteurised milk cheese as well, possibly due to cross- contamination (Fereydouni and Darbouy, 2015; Callon et al., 2016; Cardoso and Marin, 2016). It has been demonstrated that the survival capacity of STEC in the cheesemaking environment is due to the activation of stress response systems (dos Santos Rosario et al., 2021). This mechanism includes the induction of sigma factor encoded by gene *rpoS,* as a response reaction to acid stress and can also be influenced by high pressure, cold, heat, UV radiation, H_2O_2 and the concentration of salt (Cheville et al., 1996; Robey et al., 2001; Mei et al., 2015; Li et al., 2018). Other SOS response regulons were identified in UC4224 that act together with the induction of σ^S such as *gadE,* coding for one of the most efficient acid stress regulators (Vanaja et al., 2009), osmotic regulator *ompR* and oxidative stress coping gene *katG* (dos Santos Rosario et al., 2021). In line with other studies (Dineen et al., 1998), our results showed that the acidity values found in dairy products do not limit the growth of UC4224 and its three mutants being able to grow at pH 4.5, a value substantially lower than that of cheese. In a previous work by Cheng et al. (2002), where *E. coli* O157 was treated with pH 5.5 for 4 and 5 h, resulting in higher resistance to 10% NaCl and a temperature of 55°C (Cheng et al., 2002). Another study has shown that certain strains of the O157 serogroup are able to survive at low pH between 3 and 4, although the ideal condition for their growth is at pH 7 (Meira et al., 2017). The presence of these survival mechanisms in STEC explains the fact that they can be isolated from different types of dairy products and dairy-related environments. In effect, the phylogenomic analysis conducted in this study elucidated that the distribution of the *stx* genes did not follow a particular pattern in relation to the isolation sources or serogroups. The most abundant were O6, O26, O157 and O174. The latter was also found in other semi-hard raw milk cheeses as reported in previous study (Cortimiglia et al., 2021). Other studies have stated that O174 strains were sporadically isolated during outbreaks yet they represented the most frequently isolated STEC isolates from cattle and foods (Stephan et al., 2008; Lorenz et al., 2013; Cundon et al., 2018). Our results indicated a similar behaviour of the parental strains and engineered strains, demonstrating that the genomic modification did not affect the possibility to use them to study various metabolic features useful in the cheesemaking process. For the first time, we investigated the pathogenicity of O174:H2 non-LEE STEC highlighting that the virulence is related not only to *stx* genes but to other virulence factors. For this reason, further efforts should be done to gain a deeper knowledge on STEC from food regarding the importance of non-*stx* non-LEE virulence markers in defining the pathogenicity potential of dairy isolates. This work led to the creation adequate surrogates with decreased virulence for studies during food

processing. In order to enhance the suitability and safety of these strains, further experiments need to be conducted to eliminate non-*stx* virulence factors.

6. DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

7. AUTHOR CONTRIBUTIONS

GM: methodology, investigation, and writing-original draft preparation. MB: formal analysis, investigation, and writing-original draft preparation. CC and DB: writing-review and editing. PC: conceptualisation, writing-review and editing, validation, visualisation, supervision, and project administration. All authors contributed to the article and approved the submitted version.

8. FAUNDING

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10. SUPPLEMENTARY MATERIALS

Primers	Purpose	Sequence	Length	Reference
P1cat*	5' primer for replacing *stx*2 with *cat*	CTTCAGCCAAAAGGAACACCTGTATATG AAGTGTATATTATTTAAATGGG-CCATATGAATATCCTCCTTA	1117bp	This study
P2cat*	3' primer for replacing *stx*2 with *cat*	CACATACCACAAATCAGGTTATGCCTCAG TCATTATTAAACTGCACTTCA-GTGTAGGCTGGAGCTGCTTC		
Cm-F	*cat* gene	CCATATGAATATCCTCCTTA	1015bp	Serra-Moreno et al. 2006
Cm-R		GTGTAGGCTGGAGCTGCTTC		
***stx*2-F**	5' primer to verify *stx*2 deletion	GTGCCTGTTACTGGGTTTTTCTTC	118bp	Paton et al., 1993
***stx*2-R**	3' primer to verify *stx*2 deletion	AGGGGTCGATATCTCTGTCC		
P1kan*	5' primer for replacing *stx*1 with *kan*	GGTGCTCAAGGAGTATTGTGTAATATGA AAATAATTATTTTTAGAGTGCTG-GGATCCGTCGACCTGCAGT	1398bp	This study
P2kan*	3' primer for replacing *stx*1 with *kan*	GCTATTTTCACTGAGCTATTCTGAGTCAA CGAAAAATAACTTCGCTGAAT-GTGTAGGCTGGAGCTGCTTC		
PKAN F	*kan gene*	GGGATCCGTCGACCTGCAGT	1298bp	This study
PKAN R		GTGTAGGCTGGAGCTGCTTC		
***stx*1-F**	5' primer to verify *stx*1 deletion	TTCGCTCTGCAATAGGTA	555bp	Paton et al., 1995
***stx*1-R**	3' primer to verify *stx*1 deletion	TTCCCCAGTTCAATGTAAGAT		
***stx*2 Forward**	Binds *stx2a* subunit to the starting codon (external primer)	ATGAAGTGTATATTATTTA	1241bp	Muniesa et al., 2003
***stx*2 Reverse**	Binds *stx2b* subunit to the end codon (external primer)	TCAGTCATTATTAAACTG		Rüssmann et al., 1993
Cm-F stx2	Used for construction of the 5' fragment	TAAGGAGGATATTCATATGG ACGAAGATGGTCAAAACGCG	270bp	Serra-Moreno et al. 2006
Cm-R stx2	Used for construction of 3' fragment of the *cat* gene	GAAGCAGCTCCAGCCTACAC AGGAGTTAAGTATGAAGAAG	280bp	
***stx*1 start**	Binds *stx1a* subunit to the starting codon (external primer)	CCGCCTGCTATTTTCACTGA	1281bp	This Study
***stx*1 end**	Binds *stx1b* subunit to the end codon (external primer)	GGTGCTCAAGGAGTATTGTG		

*Tm= 50°C

Table S1 Oligonucleotides used in this study for the construction of antibiotic resistance cassette with homologous arms and the confirmation of the correct gene replacement

Strain	Assembly	STEC	isolation category	serotype	isolation date
1429	GCA_025989075.1	stx1	Dairy product	O79:H48	03/11/2022
2206	GCA_025989065.1	stx1 and stx2	Dairy product	O175:H16	03/11/2022
09QMA299-3B	GCA_025988945.1	stx1	Dairy product	O8:H19	03/11/2022
09QMA303-1A	GCA_025988885.1	stx2	Dairy product	O2:H27	03/11/2022
09QMA311-2-3	GCA_025988955.1	stx2	Dairy product	O175:H16	03/11/2022
09QMA33-1	GCA_025988865.1	stx2	Dairy product	O6:H10	03/11/2022
09QMA47-1	GCA_025988835.1	stx1	Dairy product	O26:H11	03/11/2022
13_5821	GCA_014608415.1	stx1 and stx2	Dairy product	O157:H7	16/09/2020
13-5819	GCA_014622575.1	stx1 and stx2	Dairy product	O157:H7	16/09/2020
13-5820	GCA_014623105.1	stx1 and stx2	Dairy product	O157:H7	16/09/2020
13-5822	GCA_014609055.1	stx1 and stx2	Dairy product	O157:H7	16/09/2020
13-5823	GCA_014621915.1	stx1 and stx2	Dairy product	O157:H7	16/09/2020
13-5824	GCA_014609655.1	stx1 and stx2	Dairy product	O157:H7	16/09/2020
1890-4	GCA_025988825.1	stx1	Dairy product	O145:H28	03/11/2022
19_0255	GCA_018133045.1	stx1	Cheese	O6:H10	23/04/2021
19_0256	GCA_018133325.1	stx2	Cheese	O8:H30	23/04/2021

2011-O26	GCA_025988775.1	stx1	Dairy product	O26:H11	03/11/2022
2026-O145	GCA_025988785.1	stx2	Dairy product	O145:H28	03/11/2022
2039-O26	GCA_025988765.1	stx1	Dairy product	O26:H11	03/11/2022
2048-O145	GCA_025988725.1	stx2	Dairy product	O145:H28	03/11/2022
2149-O103	GCA_025988735.1	stx1	Dairy product	O103:H11	03/11/2022
2236-3b	GCA_026135185.1	stx1	Dairy product	O26:H11	11/11/2022
2918-O145	GCA_025988695.1	stx2	Dairy product	O145:H28	03/11/2022
3273-O103	GCA_026135215.1	stx1	Dairy product	O103:H11	11/11/2022
3313-O111	GCA_025988665.1	stx1	Dairy product	O111:H8	03/11/2022
3382-O103	GCA_026134445.1	stx1	Dairy product	O103:H11	11/11/2022
3383-O26b	GCA_026135105.1	stx1	Dairy product	O26:H11	11/11/2022
3430-O103	GCA_026135085.1	stx1	Dairy product	O103:H11	11/11/2022
429-O26	GCA_025988635.1	stx1	Dairy product	O26:H11	03/11/2022
4712-O26	GCA_026135005.1	stx1	Dairy product	O26:H11	11/11/2022
4747-O26	GCA_025988675.1	stx1	Dairy product	O26:H11	03/11/2022
97HMPL449	GCA_025988625.1	stx1	Dairy product	O3:H12	03/11/2022
97HMPL473	GCA_025988605.1	stx1	Dairy product	O110:H9	03/11/2022
97HMPL650	GCA_025988535.1	stx1	Dairy product	O112ac:H19	03/11/2022
97HMPL652	GCA_025988575.1	stx1	Dairy product	O17:H18	03/11/2022
97HMPL657	GCA_026134525.1	stx1	Dairy product	O5:H9	11/11/2022

97HMPL915	GCA_025988525.1	stx2	Dairy product	O110:H9	03/11/2022
98HMPL324	GCA_026134235.1	stx2	Dairy product	O5:H9	11/11/2022
98HMPL325	GCA_025988545.1	stx1	Dairy product	O110:H9	03/11/2022
98HMPL475	GCA_025988505.1	stx1	Dairy product	O91:H14	03/11/2022
98HMPL479	GCA_025988485.1	stx1 and stx2	Dairy product	O136:H12	03/11/2022
98HMPL487	GCA_025988425.1	stx1	Dairy product	O174:H21	03/11/2022
BUR-FI-2013-MI-00677-1	GCA_012140015.1	stx1 and stx2	Dairy product	O157:H7	01/08/2018
BUR-FI-2013-MI-00677-7	GCA_012141065.1	stx1 and stx2	Dairy product	O157:H7	01/08/2018
BUR-FI-2013-MI-00727-1	GCA_012140585.1	stx1 and stx2	Dairy product	O157:H7	01/08/2018
BUR-FI-2013-MI-00727-6	GCA_012140105.1	stx1 and stx2	Dairy Milk	O157:H7	31/07/2018
BUR-FI-2013-MI-00758-4-1	GCA_012140765.1	stx1 and stx2	Dairy product	O157:H7	01/08/2018
BUR-FI-2013-MI-00758-4-6	GCA_012140565.1	stx1 and stx2	Dairy product	O157:H7	01/08/2018
CAL-FD-2013-MI-0636-1	GCA_012140745.1	stx1 and stx2	Dairy Milk	O157:H7	31/07/2018
CAL-FD-2013-MI-0670-1	GCA_012139765.1	stx1 and stx2	Dairy product	O157:H7	01/08/2018
CDPHFDLB-F1602047-001	GCA_012690685.1	stx1 and stx2	Dairy Milk	O?:H7	08/03/2016
CFSAN051526	GCA_012049985.1	stx1	Cheese	O6:H10	30/11/2018

CFSAN051527	GCA_012049785.1	stx1	Cheese	O6:H10	30/11/2018
CFSAN051531	GCA_012050045.1	stx2	Cheese	O8:H30	30/11/2018
CFSAN051533	GCA_012050125.1	stx1	Cheese	O6:H10	30/11/2018
CFSAN051535	GCA_012643025.1	stx1	Cheese	O6:H10	24/07/2016
CFSAN051537	GCA_012643005.1	stx1	Cheese	O6:H10	24/07/2016
EC14	GCA_025988305.1	stx2	Dairy product	O113:H4	03/11/2022
EC15	GCA_010499655.1	stx1	Cheese	O6:H10	13/02/2020
EC872416	GCA_010179635.1	stx1	Cheese	O6:H10	05/02/2020
ECA15	GCA_026134185.1	stx1 and stx2	Dairy product	O76:H19	11/11/2022
ECA34	GCA_026134195.1	stx1	Dairy product	O79:H48	11/11/2022
ECA36	GCA_026134485.1	stx1	Dairy product	O103:H2	11/11/2022
ECA37	GCA_026134765.1	stx1	Dairy product	O103:H11	11/11/2022
ECA89	GCA_026134405.1	stx1	Dairy product	O174:H21	11/11/2022
ECA97	GCA_025988125.1	stx2	Dairy product	O177:H25	03/11/2022
FDA1110442-S008-001	GCA_015329005.1	stx1	Cheese	O?:H40	07/06/2019
FDA1110442-S008-002	GCA_015328765.1	stx1	Cheese	O?:H40	12/06/2019
FDA1110442-S008-003	GCA_015328865.1	stx1	Cheese	O?:H40	07/06/2019
FDA1110442-S008-005	GCA_015328745.1	stx1	Cheese	O?:H40	12/06/2019
FLAG-59726	GCA_012884835.1	stx2	Cheese	O172:H5	12/03/2020

MOD1-EC2541	GCA_002516325.1	stx1	Cheese	O6:H10	11/10/2017
MOD1-EC2549	GCA_002514885.1	stx1	Cheese	O6:H10	11/10/2017
MOD1-EC2766	GCA_002476375.1	stx1	Cheese	O6:H10	06/10/2017
MOD1-EC2769	GCA_002476355.1	stx1	Cheese	O81:H21	06/10/2017
MOD1-EC3827	GCA_002516025.1	stx1	Cheese	O20:H16	11/10/2017
MOD1-EC3831	GCA_002515965.1	stx1	Cheese	O20:H16	11/10/2017
PNUSAE004169	GCA_012494245.1	stx1	Cheese	O?:H5	02/03/2017
PNUSAE004170	GCA_012494445.1	stx2	Cheese	O?:H48	02/03/2017
PNUSAE004171	GCA_012494465.1	stx1	Cheese	O5:H9	02/03/2017
PNUSAE004172	GCA_012494365.1	stx1	Cheese	O5:H9	02/03/2017
PNUSAE004173	GCA_012494405.1	stx1	Cheese	O5:H9	02/03/2017
PNUSAE063752	GCA_015226665.1	stx1 and stx2	Dairy Milk	O157:H7	04/11/2020
PNUSAE068927	GCA_017734475.1	stx1 and stx2	Cheese	O38:H21	17/03/2021
PNUSAE113785	GCA_025308195.1	stx2	Cheese	O157:H7	16/09/2022
PSU-3645	GCA_017946745.1	stx1	Cheese	O121:H7	13/04/2021
SC_872416	GCA_015159835.1	stx1	Cheese	O6:H10	01/11/2020
SC_886340	GCA_015159895.1	stx2	Cheese	O8:H30	01/11/2020
Slk8430767	GCA_025988085.1	stx2	Dairy product	O157:H7	03/11/2022
UC4128	GCA_016123515.1	stx1 and stx2	Cheese	O174:H2	19/12/2020

UC4129	GCA_016123475.1	stx2	Cheese	O116:H48	19/12/2020
UC4130	GCA_016123455.1	stx1 and stx2	Cheese	O174:H2	19/12/2020
UC4131	GCA_016123425.1	stx1 and stx2	Cheese	O174:H2	19/12/2020
UC4132	GCA_016123415.1	stx1 and stx2	Cheese	O174:H2	19/12/2020
UC4133	GCA_016123385.1	stx2	Cheese	O116:H48	19/12/2020
UC4134	GCA_016123375.1	stx2	Cheese	O116:H48	19/12/2020
UC4224	GCA_025369975.1	stx1 and stx2	Cheese	O174:H2	22/09/2022
VA-WGS-00283	GCA_012830355.1	stx2	Cheese	O157:H7	28/10/2014
VL1029a	GCA_015117575.1	stx2	Dairy Milk	O157:H43	05/10/2020

Table S2 Dairy associated Escherichia coli genomes used to infer phylogeny.

Gene	Subtype	Molecule	Position in con	Protein function
Shiga Toxins				
stx1A	STEC	Chromosome	712684-713631	Shiga toxin 1, subunit A, variant a
stx1B	STEC	Chromosome	712405-712674	Shiga toxin 1, subunit B, variant a
stx2A	STEC	Chromosome	4855779-4856738	Shiga toxin 2, subunit A, variant a
stx2B	STEC	Chromosome	4856750-4857019	Shiga toxin 2, subunit B, variant a
LAA PAI				
ag43	LAA PAI related	Chromosome	1911034-1914153	ORF67 that promotes autoaggregation
hes	LAA PAI related	Chromosome	1842328-1843068	Hemagglutinin from STEC
lesP	LAA PAI related	Chromosome	1866536-1870627	LAA encoded SPATE
pagC	LAA PAI related	Chromosome	1881900-1882475	Serum resistance
sisA	LAA PAI related	Chromosome	1839242-1840285	shiA-like inflammation suppressor genes A
Other VFs				
hra	adherence	Chromosome	1842328-1843082	Heat-resistant agglutinin
iss	serum resistance	Chromosome	4166610-4166903	Increased serum survival
lpfA	adherence	Chromosome	2800504-2801076	Long polar fimbriae
ompT	adherence	Chromosome	4194798-4195751	Outer membrane protease
papC	adherence	Chromosome	3397513-3400023	Outer membrane usher P fimbriae
non-LEE T3SS effectors				
bipA	transcription	Chromosome	2951208-2953031	50S ribosomal subunit assembly factor BipA
cpxP	transcription	Chromosome	2997993-2998493	Periplasmic protein CpxP
cpxA	transcription	Chromosome	2995775-2997148	Sensor histidine kinase CpxA
cpxR	transcription	Chromosome	2997145-2997843	Transcriptional regulatory protein CpxR
fis	transcription	Chromosome	2299433-2299729	DNA-binding protein Fis
gadE	transcription	Chromosome	2533254-2533781	acid resistance transcriptional activator GadE
hha	transcription	Chromosome	4067077-4067295	Hemolysin expression-modulating protein Hha
hns	transcription	Chromosome	4997804-4998217	DNA-binding protein H-NS
ihfB	transcription	Chromosome	4619240-4619524	Integration host factor subunit beta
sspA	transcription	Chromosome	2256653-2257291	Stringent starvation protein A
eutR	transcription	Chromosome	1286916-1287968	HTH-type DNA-binding transcriptional activator EutR
leuO	transcription	Chromosome	3658723-3659667	HTH-type transcriptional regulator LeuO
sdiA	transcription	Chromosome	772807-773529	Regulatory protein SdiA
clpX	post-transcriptional	Chromosome	4044415-4045689	ATP-dependent Clp protease ATP-binding subunit Clp
clpP	post-transcriptional	Chromosome	4043666-4044289	ATP-dependent Clp protease proteolytic subunit
degP	post-transcriptional	Chromosome	3754780-3756204	Periplasmic serine endoprotease DegP
DsrA	post-transcriptional	Chromosome	799931-800017	DsrA RNA
rpoS	regulation	Chromosome	1573744-1574736	RNA polymerase sigma factor RpoS
kdpE	Quorum sensing	Chromosome	4342543-4343220	KDP operon transcriptional regulatory protein KdpE
qseC	Quorum sensing	Chromosome	1999173-2000522	Sensor protein QseC
qseE	Quorum sensing	Chromosome	1415709-1417091	two component system sensor histidine kinase QseE/G
qseG	Quorum sensing	Chromosome	1414831-1415544	Quorum-sensing regulator protein G
qseF	Quorum sensing	Chromosome	1413507-1414841	two-component system response regulator QseF/GlrR
rcsB	Quorum sensing	Chromosome	1071336-1071986	transcriptional regulator RcsB
GlmY_tke1	Quorum sensing	Chromosome	1417231-1417378	Glm Y RNA activator of glmS mRNA
GlmY_tke1	Quorum sensing	Chromosome	2874402-2874550	Glm Y RNA activator of glmS mRNA
GlmZ_SraJ	Quorum sensing	Chromosome	1417193-1417380	GlmZ RNA activator of glmS mRNA
GlmZ_SraJ	Quorum sensing	Chromosome	2874400-2874606	GlmZ RNA activator of glmS mRNA
plasmid encoded virulence				
cia	colicin	pUC4224_1	109510-109762	Colicin ia
espP	SPATE	pUC4224_2	27625-31527	autotransporter, serine protease
ehxA	hemolysin	pUC4224_1	6244-9240	hemolysin transport protein
ehxB	hemolysin	pUC4224_1	4074-6191	hemolysin transport protein
ehxC	hemolysin	pUC4224_1	9242-9757	hemolysin transport protein
ehxD	hemolysin	pUC4224_1	2631-4070	hemolysin transport protein
iha	adherence	pUC4224_1	38300-40387	Adherence protein
traT	serum resistance	pUC4224_1	109940-110673	Outer membrane protein complement resistance
celb	colicin	pUC4224_1	6118-6261	Endonuclease colicin E2

Table S3 Distribution of virulence factors in UC4224

	pH 4						pH 4.5						pH 5					
concentration (CFU/ml)	10^9	10^8	10^7	10^6	10^5	10^4	10^9	10^8	10^7	10^6	10^5	10^4	10^9	10^8	10^7	10^6	10^5	10^4
UC4224	-	-	-	-	-	-	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++
UC4176	-	-	-	-	-	-	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++
UC4177	-	-	-	-	-	-	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++
UC4178	-	-	-	-	-	-	+++	+++	-	-	-	-	+++	+++	+++	+++	+++	+++

"-" corresponds to no growth. "+++" corresponds to growth.

Table S4 Growth values of UC4224 and mutants UC4176 (Δstx1), UC4177 (Δstx2) and UC4178 (Δstx1,Δstx2) during treatment with lactic acid at pH 4, 4.5 and 5.

9 CFU/10 µl	**UC4224**	**UC4176*(Δstx1)***	**UC4177*(Δstx2)***	**UC4178*(Δstx1Δstx2)***
UC4224	-	<0,0001	<0,0001	<0,0001
UC4176*(Δstx1)*	<0,0001	-	<0,0001	<0,0001
UC4177*(Δstx2)*	<0,0001	<0,0001	-	0,1106
UC4178*(Δstx1Δstx2)*	<0,0001	<0,0001	0,1106	-
18 CFU/10 µl				
UC4224	-	<0,0001	<0,0001	<0,0001
UC4176*(Δstx1)*	<0,0001	-	<0,0001	<0,0001
UC4177*(Δstx2)*	<0,0001	<0,0001	-	0,151
UC4178*(Δstx1Δstx2)*	<0,0001	<0,0001	0,151	-
180 CFU/10 µl				
UC4224	-	<0,0001	0,0006	0,0013
UC4176*(Δstx1)*	<0,0001	-	<0,0001	<0,0001
UC4177*(Δstx2)*	0,0006	<0,0001	-	0,7832
UC4178*(Δstx1Δstx2)*	0,0013	<0,0001	0,7832	-
1800 CFU/10 µl				
UC4224	-	0,0003	>0,9999	>0,9999
UC4176*(Δstx1)*	0,0003	-	0,0003	0,0003
UC4177*(Δstx2)*	>0,9999	0,0003	-	>0,9999
UC4178*(Δstx1Δstx2)*	>0,9999	0,0003	>0,9999	-

Table S5.1 Statistical significance difference determined using the log-rank test between tested strains at different injection doses a) 9 CFU/10µl b) 18 CFU/10µl c) 180 CFU/10µl d) 1800 CFU/10µl. Highlighted in grey the statistically significant differences.

Strains	**LD_{50} CFU/10 µl**	**95% CI**		**R^2**
		Lower	**Upper**	
UC4224	6	3.39	10.36	0.988
UC4176*(Δstx1)*	81,7	35.85	185.94	0.986
UC4177*(Δstx2)*	50.5	20.45	124.55	0.933
UC4178*(Δstx1Δstx2)*	582.7	142.53	2381.78	0.947

Table S5.2 LD50 detected for the four tested strains, with the respective 95% Interval of Confidence (CI) and coefficient of determination (R^2).

CHAPTER 6

Taxonomical Identification and Safety Characterization of *Lactobacillaceae* from Mediterranean Natural Fermented Sausages.

Taxonomical Identification and Safety Characterization of *Lactobacillaceae* from Mediterranean Natural Fermented Sausages.

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1. ABSTRACT

Fermented meat products represent an important industrial sector in Europe, particularly in the Mediterranean Countries (MC), where the presence of numerous local productions, still obtained through spontaneous fermentation, is recognized as a formidable treasure chest of unexplored microbial biodiversity. *Lactobacillaceae* naturally occurring in fifteen spontaneously fermented sausages from MC (Italy, Spain, Croatia, and Slovenia) were isolated and taxonomically characterized using molecular techniques. Additionally, a safety assessment for the presence of antibiotic resistances and biogenic amine (BA) production was performed to determine their suitability as autochthonous starter cultures. Molecular typing, performed using REP-PCR, discriminated 151 strains belonging to *Latilactobacillus sakei* (59.6%), *Latilactobacillus curvatus* (26.5%) and *Companilactobacillus alimentarius* (13.9%). The minimum inhibitory concentrations (MICs) of eight different antibiotics revealed a high resistance to streptomycin (27%), tetracycline (16%), followed by gentamycin (14%) and kanamycin (13%). Interestingly, the results showed a geographical distribution of resistant biotypes. *tetM*/*tetS* or *ermB* genes were identified in only six strains. The amino-biogenic potential of the strains was assessed, confirming the absence of this trait among *L. sakei*, while a high number of producer strains was found among *L. curvatus*. On the 151 analyzed strains, 45 demonstrated safety traits for their future use as starter food cultures. These results open the way to further studies on the technological properties of these promising autochthonous strains, strongly linked to the Mediterranean environment.

2. INTRODUCTION

The curing of meats can be considered one of the most ancient methods for preserving perishable raw materials and the origin of this approach dates back several centuries [1,2]. Among cured meats, dry sausage preparation combines the use of curing salts with a fermentation step that involves several microorganisms including lactic acid bacteria (LAB), staphylococci, micrococci and fungi [3,4]. Early studies concerning the complex dynamics of this microbial process were published about 60 years ago [5,6] and lead to establish criteria f or the selection of starter cultures to be used for driving meat fermentation [7,8]. Nowadays, the use of starter cultures is common in industrial products, and they are mainly constituted of LAB and coagulase negative cocci (CNC). Among LAB, the strains mainly selected belong to the species *Latilactobacillus sakei*, *Latilactobacillus curvatus*, *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus* and *Pediococcus acidilactici* [9]. However, as observed by [2], the use of starters may result in the loss of microbial biodiversity and, therefore, of the peculiar characteristics (impoverishment of sensory features), if compared to artisanal sausages obtained with spontaneous fermentation. On the other hand, selected cultures are able to guarantee the constant quality, safety and longer shelf-life [2,10]. The search for new and tailor-made cultures able to impart specific and traditional attributes to fermented sausages represents an important approach to overcome the negative aspects and preserving the authenticity and recognisability of artisanal products. In this perspective, the presence of numerous local products still

obtained through spontaneous fermentation is a formidable source of unexplored microbial biodiversity of a given terroir which could be exploited for isolating new starter candidates [11–13].

The most peculiar traits of LAB strains in meat fermentation are undoubtedly their abilities to rapidly decrease the pH and to colonize the environment throughout the complete production process [14]. A low pH limits the growth of undesirable species (pathogens and spoilers) and favours texture and water loss by approaching the isoelectric point of meat proteins [15]. However, further technological characteristics are requested for their use as meat starter cultures. These features can represent an important trait for starter selection also in the frame of NaCl reduction, which characterizes the new market trends considering nutritional needs and consumer demands [16]. Generally, LAB starters are selected in order to improve safety and reduce hygienic and toxicological risks in food [2,17], but they have to be firstly safe, so that their use as starter cultures for fermentation requires a safety assessment. Biogenic amines (BA) are toxic products deriving from amino acid decarboxylation that accumulate in sausages during fermentation and ripening [18]. Many bacterial species may contribute to their production in fermented foods, including LAB. For this reason, the absence of specific decarboxylases is a prerequisite for LAB used as starter cultures, mainly because their performances could affect BA accumulation during ripening [19,20]. The presence of genetic clusters containing the necessary genes for BA production have been deeply studied, especially for the most dangerous BA, i.e., tyramine and histamine [21,22]. The conditions of BA accumulation define the so called aminobiogenic potential, that can be tested both genetically and phenotypically [23]. Another relevant safety aspect is the presence of antibiotic resistance genes in mobile genetic elements, such as plasmids and transposons. In fact, these elements can be transferred to other species, including pathogenic bacteria, during food manufacture or during the passage through the gastrointestinal tract [24]. This poses an additional risk due to the nature of consumption of ready-to-eat fermented products and their potential to become strong antibiotic resistance reservoirs [25]. Particularly, the presence of tetracycline and erythromycin resistant lactobacilli, studied applying EFSA (European Food Safety Authority (EFSA) 2012) cut-off limits, has been well documented in fermented dry sausages produced in northern Italy [26,27].

The aim of this study was the isolation, characterization and safety assessment of autochthonous *Lactobacillaceae* from 15 Mediterranean spontaneously fermented sausages, collected from four different MC (Italy, Spain, Croatia, and Slovenia) and previously characterized for their characteristics and bacterial biodiversity [28]. This investigation had the purpose to widen the previously studied microbiota composition, in order to understand the ecology of these natural fermented meats and to know which LAB species are the most abundant. In addition, the work was aimed to study the presence and the type of strain antimicrobial resistances and aminobiogenic potential. With this aim, more than 900 isolates have been genotyped using fingerprint analysis for the differentiation of the strains, which have been further taxonomically identified and characterized for their safety features. This knowledge will be the starting point to further determine strains suitability to be used in foods from one side as potential

autochthonous starter cultures, studying their technological properties, and secondary, as protective food cultures against pathogenic and spoiling agents, assessing their antimicrobial potential.

3. MATERIALS AND METHODS

3.1. Sausage Samples and *Lactobacillaceae* Isolation

A sampling of 15 natural-fermented sausages, produced without any starters addition, was collected at the end of ripening from four different MC and particularly: three sausages were obtained from Italy (IM1, IM2, IAL), two from Slovenia (SN, SWO), seven from Spain (ESA, ESB, ESE, ESO, ECB, ECE, ECO) and three from Croatia (HNS, HS, HZK).

Times and ripening conditions were heterogeneous and only for the three Slovenian domestic samples, smoking was used (14 days). These samples were previously characterized for their chemical-physical features and their microbial profile [28].

For cultivation-dependent analysis, sausages were processed as previously described by Barbieri et al. [28]: Man-Rogosa-Sharpe (MRS) agar medium (Oxoid, Milan, Italy) was employed for presumptive LAB counts and their isolation at 30 °C for 48 h in anaerobic conditions achieved using Anaerocult A (Merck, Darmstadt, Germany) in anaerobic jars. Grown colonies on MRS agar plates were randomly selected, picked with a sterile loop, and streaked onto new MRS plates in duplicate. Isolates were collected from plates containing from 20 to 50 colonies. A minimum of 22 to a maximum of 70 isolates for each sample was considered. The pure cultures obtained were observed for morphological characteristics and tested by means of catalase test, Gram test, growth at 15 °C and 45 °C, as well as for their homo or heterolactic fermentation. For further analyses, the isolates were stored at −20 °C in MRS broth containing 20% glycerol (Carlo Erba, Milan, Italy).

3.2. DNA Extraction and REP-PCR Analysis

All isolates were cultured in 10 mL of MRS broth (GranuCult®, Darmstadt, Germany), incubated at 30 °C overnight. From these fresh pure cultures, isolates were streaked in MRS agar and incubated in anaerobic conditions for 48 h. Single colonies were selected from the agar plates to perform DNA extraction using the fast microLYSIS®-Plus DNA extraction kit (Microzone, Labogen, Stourbridge, UK) following the manufacturer's instructions; 20 µL of DNA was obtained and used for the molecular analysis. The rep-PCR (Repetitive element (or extragenic) palindromic-Polymerase Chain Reaction) using oligonucleotide primer (GTG) 5 (5^0–GTGGTGGTGGTGGTG–3^0), was chosen for fingerprint analysis of isolates [29]. The amplified products were electrophoresed in a 2.5% agarose gel. The study was performed analysing the fingerprint profile for each group of isolates from the different sausage types. The selected biotypes were grown in 10 mL of MRS broth and incubated overnight at 30 °C under anaerobic conditions. Cells were collected by centrifugation (3200× *g*, 15 min) and frozen at −20 °C in MRS + glycerol 20% solution.

3.3. Genotyping Identification of Isolates

16S rRNA gene sequencing was done on all biotypes with different rep-PCR profiles using specific primers and PCR reaction designed by [30]. After amplification and before sequencing, the PCR products were purified using ExoSAP-IT™ (Applied Biosystems™, Thermo Fisher Scientific, Leicestershire, UK) according to the protocol provided by the manufacturer. The DNA was sequenced by a commercial facility (Eurofins Genomics, Italy) and the obtained sequences were analysed using the Ribosomal Database Project tools (http://rdp.cme.msu.edu/, accessed on 1 Febraury 2021) and assigned to the species with the highest percentage of identity. Species-specific PCR reactions for the identification of *Latilactobacillus sakei* and *Latilactobacillus curvatus* were performed on those isolates that were not correctly assigned to species level (identity ≤ 98.7% [31]). PCR products were separated by electrophoresis in a 1% agarose gel and visualized by Sybr-Safe staining.

The relative frequency of intra-species biotypes for each MC salami sample has been calculated using Microsoft Excel 2016, Version 2207.

3.4. Antimicrobial Susceptibility Testing

EFSA Guidance [32] was followed for antibiotic resistance determination of the selected strains and minimal inhibitory concentration (MIC) testing. Resistances to Ampicillin (Amp), Chloramphenicol (Chl), Clindamycin (Cli), Erythromycin (Ery), Gentamicin (Gen), Tetracycline (Tet), Kanamycin (Kan) and Streptomycin (Str) were determined by using micro dilution technique in the recommended LSM medium (Iso-Sensitest™ broth 90% [Thermo Fisher Scientific, Leicestershire, UK], MRS broth 10%) [33]. Bacterial growth was measured after 48 h of incubation at 28 °C for *Latilactobacillus sakei* and 37 °C for *Latilactobacillus curvatus*, under anaerobic conditions.

Relative abundance of resistant biotypes for each species and antibiotics tested were calculated using Microsoft Excel 2016, Version 2207 (Microsoft Corporation, Redmond, WA, USA).

3.5. PCR-Based Screening of Resistance Genes

The presence of Tetracycline and Erythromycin resistance genes was screened by standard PCR with specific primers reported in Table 1. Genes coding for ribosomal protection proteins conferring Tetracycline resistance were targeted with specific primers for *tet* (*W*) [34], *tet* (*M*) [35] and tet (S) [36]. Tetracycline efflux pump gene, *tet* (*L*) was also searched using gene-specific primers [37]. The presence of Erythromycin resistance genes was tested using specific primers for *ermA*, *ermB* [38] and *ermC* [39]. PCR products were separated by electrophoresis on a 1% agarose gel and visualized by Sybr-Safe staining.

Table 2 Primers for selected antibiotic resistance genes used in this study

Primers Name	Oligonucleotide Sequence (5^0-3^0)	Expected Band (bp)	Positive Control Strain	Reference
ermA1 **ermA2**	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAGT	645	*E. faecium* PE1	[38]
ermB1 **ermB2**	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	639/694	*E. faecium* PE1	[38]
ermC1 **ermC2**	ATCTTTGAAATCGGCTCAGG CAAACCCGTATTCCACGATT	275/294	*L. reuteri* 70	[39]
tetL1 tetL2	GTMGTTGCGCGCTATATTCC GTGAAMGRWAGCCCACCTAA	696	*E. faecium* LMG 20927	[29]
tetM1 tetM2	GAACTCGAACAAGAGGAAAGC ATGGAAGCCCAGAAAGGAT	740	*L. plantarum* 146	[35]
tetS1 tetS2	GGAGTACAGTCACAAACTCG GGATATAAGGAGCAACTTTG	335	*L. reuteri* 541	[36]
tetW1 tetW2	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	168	*L. reuteri* 534	[34]

3.6. Biogenic Amines (Bas) Production

To test the aminobiogenic potential of the strains, overnight cultures on MRS were inoculated to a concentration of 6 log CFU/mL in Bover-Cid and Holzapfel broth, supplemented with the BA precursors (histidine, tyrosine, ornithine or lysine) and incubated at 30 °C. The ability to produce BAs was assessed with the method proposed by Bover-Cid and Holzapfel (1999) and BA production confirmation was performed thought HPLC according to the method reported by [28].

4. RESULTS AND DISCUSSION

4.1. Strain Genotyping and Identification of Biotypes

A total of 914 microorganisms, grown on MRS agar medium and presumptive classified as LAB based on Gram staining and catalase test results, were isolated from 15 naturalfermented sausages from MC: 173 isolates from Italian sausages, 140 from Slovenian sausages, 444 from Spanish sausages and 157 from Croatian sausages, respectively. To achieve taxonomical identification at the strain level, (GTG)5-rep-PCR fingerprinting technique was applied on DNA extracted from the all the isolated samples. Representative profiles for each sausage were selected and subjected to partial 16S rRNA gene sequencing and species-specific PCR for *L. sakei* and *L. curvatus*. In Table 2 the total number of isolates and biotypes per each type of MC sausage are reported.

Table 3 Type of sausages, number of isolates and biotypes from the four different production countries (Italy, Slovenia, Spain and Croatia).

Production Country	Type of Sausage	Sample Name	Number of Isolates	Biotypes
Italy	Salame Fabriano-producer 1 (Marche)	IM1	58	3
	Salame Fabriano-producer 2 (Marche)	IM2	48	2
	Salame Alfianello (Brescia), Lombardy	IAL	67	4
Slovenia	Traditional smoked salami with nitrates	SN	70	5
	Traditional smoked salami without nitrates	SWO	70	6
Spain	Salchichòn Bérchules	ESB	69	11
	Chorizo Bérchules	ECB	48	16
	Chorizo Olvera	ECO	52	7
	Salchichon Olvera	ESO	70	19
	Chorizo Ecija	ECE	69	22
	Salchichon Ecija	ESE	69	8
	Salchichon Alhendin	ESA	67	11
Croatia	Salami ZminjskaKlobasica	HZK	53	21
	Traditional smoked salami	HS	49	5
	Traditional unsmoked salami	HNS	55	11
Total	15		914	151

Based on the data described in Table 2, a total of 151 biotypes were detected. The fingerprint analysis showed a higher variability in the electrophoretic profiles of the samples from Spanish and Croatian sausages. From 69 and 70 isolates of the Spanish Salchichones ESB and ESO, 11 and 19 different biotypes have been respectively differentiated, while out of 48 and 69 isolates from the Spanish Chorizo ECB and ECE, 16 and 22 biotypes have been highlighted. Regarding Croatian samples, the richest in LAB biodiversity was the Salami HZK, with 21 biotypes out of 53 isolates. Differently, Italian and Slovenian sausages were characterized by a lower biodiversity, with only two identified biotypes among 48 isolates from Italian Salame IM2, 5 biotypes among 70 isolates from the Slovenian traditional smoked salami SN and 6 biotypes among 70 isolates from the Slovenian traditional smoked salami SWO.

Secondarily, the 151 strains that showed unique rep-PCR profiles, were identified by 16S rRNA gene sequencing. Isolates that failed to be assigned to any species (level of identity ≤ 98.7%), were identified using species-specific PCR for the identification of *L. sakei* and *L. curvatus*. The combined molecular approach allowed to identify three dominant species: *L. sakei*, *L. curvatus* and *C. alimentarius*; particularly, 90 strains were assigned to *L. sakei*, 40 strains to *L. curvatus* and 21 strains to *C. alimentarius*. As frequently stated by previous works [40,41], natural meat fermentation is dominated by coagulase-negative cocci (CNC) and LAB, whose most commonly species are represented by *L. sakei*, *L. curvatus* and *L. plantarum*. Considering the results of the combined methodology to assess the taxonomic identity of the 150 *Lactobacillaceae* biotypes, *L. sakei* resulted to be the dominant species in the IM1 (100%), IM2 (100%), IAL (75%), SN (100%), SWO (100%), ESB (91%), ESE (62.5%), ESA (82%), HZK (81%), HS (100%), HNS (64%) sausages. *L. curvatus* prevailed in the ECE (63.7%) sausages, while the ESO sausages presented an equal presence of *L. sakei* and *L. curvatus* (47% each). *C. alimentarius* dominated in the ECB (87.5%) and in the ECO (57%) sausages.

These data confirmed the outcome obtained through metagenomics analysis on the same samples [28], where *L. sakei* was the dominant species among LAB, especially in IM2, IAL, and SN samples, followed by members of the *L. sakei* group in lower amounts. This dominance is highlighted also by the relative frequency of the *L. sakei* biotypes that were found in higher percentage particularly in the Italian and Slovenian sausages, followed by Croatian ones; a higher species biodiversity was instead typical of Spanish samples, where the species distribution among biotypes is more balanced for the three described LAB (Figure 1 and Supplementary Material Table S1).

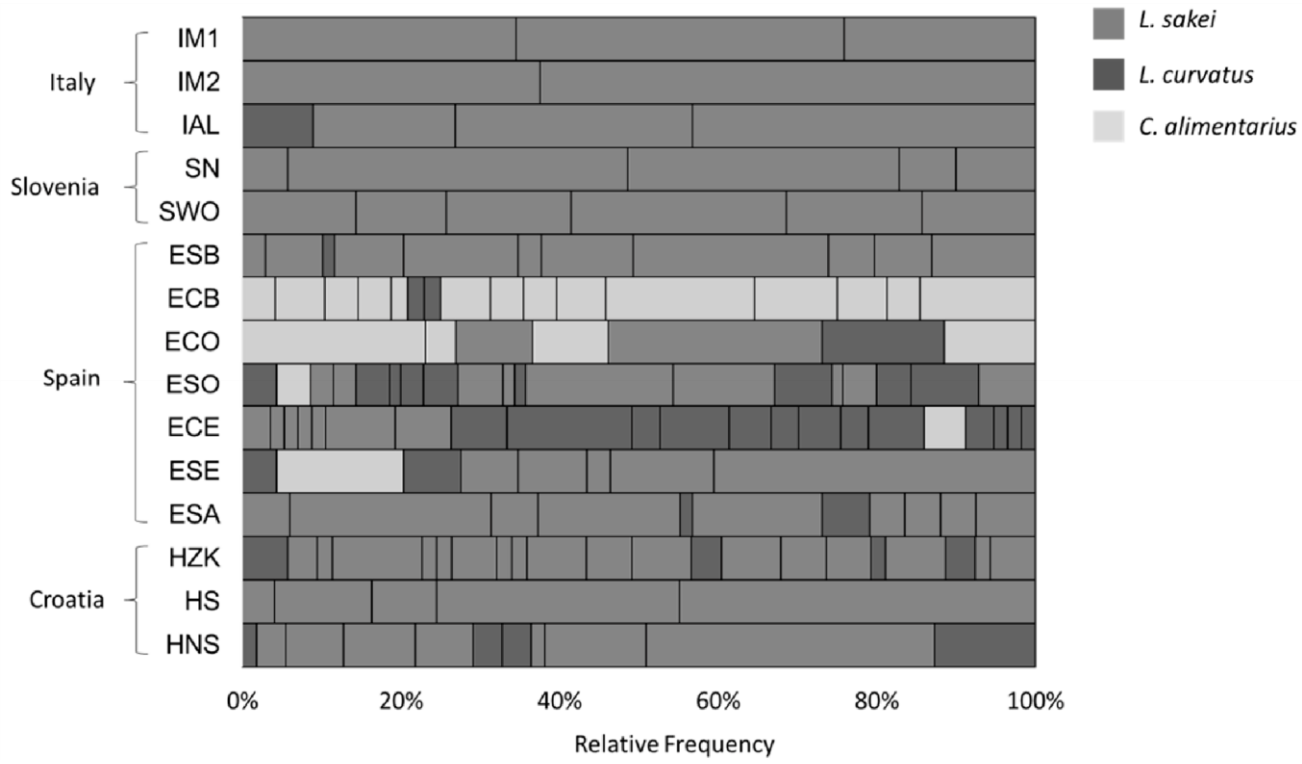

Figure 7 Relative frequency of LAB biotypes, identified as L. sakei, L. curvatus and C. alimentarius, found in each MC fermented sausages.

During the ripening process of natural fermented sausages, LAB species diversity is limited and L. sakei is one of the main adapted species to the restrictive conditions generally present in the dry meat environment, due to the species excellent adaptation, competitiveness and assertiveness in the meat matrix [12,42]. The samples of the study were processed at the end of the maturation period when low availability of sugars was still supposed to be present in the sausages and free amino acids were probably used by *L. sakei* to grow and survive in these conditions. This species is highly adapted to this ecological niche, due to its metabolic pathways, including the arginine deiminase pathway and the utilization of nucleosides [12,43,44]. The Spanish sausages surprisingly showed a high percentage of biotypes belonging to the *C. alimentarius* species; sample ECB showed, for example, 95% *C. alimentarius* biotypes. *C. alimentarius* species was also previously highlighted through amplicon sequencing and metagenomic analysis in these Mediterranean sausage samples [28]. In fact, also in this preliminary work, high quantities of the members of *C. alimentarius*, *C. heilongjiangensis* and *C. versmoldensis* were present in many Spanish sausages, in particular ESE and ECB (55.3 and 45.0% of the total ASVs, respectively). This species has been reported as a regional peculiarity in the literature [45,46], but its presence was described also as minoritarian in some traditional fermented salami of Southern Italy, such as Naples-type salami [47].

In addition, the Spanish sausages, if compared to the other MC fermented meats, had a higher strain biodiversity in terms of different identified biotypes. The technological parameters, together with the ingredients, the fermentation process and ripening conditions could strongly influence the survival and adaptation of different bacterial populations to a peculiar environment.

4.2. Safety Assessment of Isolated Strains

Starter strains to be used as food cultures have to comply with safety criteria such as the absence of antibiotic resistance genes and the incapacity to produce biogenic amines [48,49]. In this context, the two main occurring species *L. sakei* and *L. curvatus* (90 and 39 isolates, respectively) were tested for their safety features in order to prove their safe use as food cultures. Particularly, their antimicrobial resistance profile was tested by micro dilution technique and the aminobiogenic potential through HPLC analysis. *C. alimentarius* strains were not analysed for the characterization, since this species is not considered among the possible adequate starter cultures in meat productions.

4.3. Antibiotic Resistance Assessment

Cut off values established by EFSA [32], were used as reference to search for the presence of resistant biotypes isolated from the 15 artisanal fermented sausages. A unimodal distribution of the MICs values, divided per species, is reported in Table 3 for all the analysed samples.

Table 4 Unimodal distribution of MICs for L. sakei and L. curvatus isolated biotypes. The resistant strains for each antibiotic are highlighted (bold and italics). Gen = Gentamycin; Kan = Kanamycin; Str = Streptomycin; Tet = Tetracycline; Ery = Erythromycin; Clin = Clindamycin; Chlor = Chloramphenicol; Amp = Ampicillin.

Antibiotic[a]	Species	Isolates with the following mics (μg ml^{-1})													
		<0,016	**0.032**	**0.063**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
GEN	*Lat. sakei*						1	1	4	11	33	27	10	3		
	Lat. curvatus						1	3	2	9	10	10	5			
KAN	*Lat. sakei*								1	5	12	20	20	21	5	6
	Lat. curvatus								1	3	8	11	7	7	3	
STR	*Lat. sakei*								1	5	10	10	17	22	22	3
	Lat. curvatus							1		2	3	7	7	9	4	7
TET	*Lat. sakei*				7	5	9	26	17	5	6	1		14		
	Lat. curvatus					2	4	3	9	9	6	6	1			
ERY	*Lat. sakei*	4	11	37	17	13	3	5								
	Lat. curvatus		3		7	15	7	6	2							
CLIN	*Lat. sakei*		66	6	1	9	2	2	2	1	1					

	Lat. curvatus	20	3	11	1		2	2	1		
CHLOR	*Lat. sakei*			7	2	10	33	29	7	2	
	Lat. curvatus					1	4	4	27	3	1
AMP	*Lat. sakei*	7			2	9	20	12	40		
	Lat. curvatus				1	15	11	7	4	2	

Considering *L. sakei*, the most representative isolated species, 45 strains out of 90, showed resistance to at least one antibiotic. A total of 28 strains presented only 1 resistance, while a discrete number of multidrug resistant strains have been found; particularly, 11 strains showed 2 resistances, 5 strains carried 3 resistances and 1 strain, the ECE-5 isolated from a Spanish sausage, presented 5 resistances (data not shown). For what concerns the antibiotic classes, these results demonstrated that there is a limited susceptibility to aminoglycosides, especially for Streptomycin, with 25 resistant strains, followed by Tetracycline, with 15 resistant strains, Gentamycin, with 13 resistant strains, and by Kanamycin, with 11 resistant strains. The genotypic analysis on *L. sakei* showed the presence of two genes coding for Tetracycline resistance: *tetS* and *tetM*. *TetS* gene was detected in the strains HNS-7, HNS-3 and ESB-57, while *tetM* gene was found in HNS-3, ECO-19 and ESB-57. No resistant strains were detected for Erythromycin and Ampicillin. In the case of *L. curvatus*, 20 out of the 40 strains were recognised as resistant. Especially, 12 strains showed one resistance, only one strain presented 2 resistances, 6 strains carried 3 resistances and 1 strain, the HZK-49 isolated from a Croatian sausage showed 5 resistances. In this case, the results showed that susceptibility to aminoglycosides is the lowest; particularly, 11 strains presented resistance to Streptomycin, followed by Gentamycin, with 5 resistant strains, and by the Kanamycin, with 3 resistant strains. In addition, 7 isolates were resistant to Tetracycline, 4 isolates were resistant to Chloramphenicol, 3 strains were resistant to Clindamycin and 2 strains were resistant to Ampicillin. Furthermore, the genotypic analysis presented the gene *ermB* coding for Erythromycin resistance in *L. curvatus* strains ESO-52 and HZK-49; the results confirmed the output obtained with the micro dilution method. Comparing the two LAB dominant species for antibiotic resistance, Streptomycin resulted to be the most spread resistance both in *L. sakei* and *L. curvatus* isolates (Figure 2), followed by Tetracycline. Differently from *L. curvatus* strains, *L. sakei* isolates harboured no resistances to Ampicillin and Erythromycin.

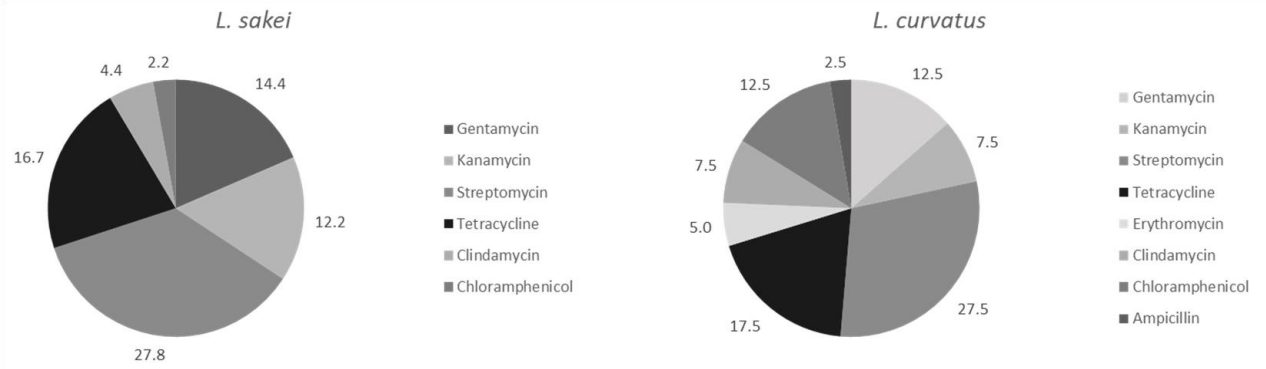

Figure 8. Relative abundance of resistant biotypes for each species and antibiotics tested.

The occurrence of strains characterized by MICs higher than EFSA breakpoints was found to be superior in the ones isolated from the Spanish and the Croatian sausage samples, showing a geographical distribution of resistant biotypes. In fact, the highest values for Gentamycin was 64 μg/mL (ESA and ESE sausages); for Kanamycin was 256 μg/mL(ESA, HZK, HS sausages); for Streptomycin was 256 μg/mL (ESO, ECE, ESE, HZK, HNS sausages); for Tetracycline was 64 μg/mL (ESB, ECO, ESO, ECE, HZK and HNS sausages); for Erythromycin was 2 μg/mL (ESO, HZK sausages); for Clindamycin was 8 μg/mL (HS sausage); for Chloramphenicol was 8 μg/mL (ECE and HZK sausages); for Ampicillin was 8 μg/mL (ESO and HNS sausages). Among the antibiotics, Streptomycin, Tetracycline, Gentamycin and Kanamycin resistances were the most detected: 27% of the strains were resistant to Streptomycin, 16% to Tetracycline, 14% to Gentamycin and 10% to Kanamycin. A lower number of strains were found to be resistant to other antimicrobials, in particular: 3% to Clindamycin, 3% to Chloramphenicol, 1.5% to Erythromycin and 1.5% to Ampicillin. Fermented sausages from Italy (IM1, IM2 and IAL), Slovenian sausages SN and SWO and the Spanish CB sausages showed to be colonized by susceptible lactobacilli, with no resistances to the antibiotics tested in the study.

4.4. Biogenic Amines (BAs) Production

Aminobiogenic potential results demonstrated high variability among the strains based on their species and source of isolation. No BA producers were detected among the *L. sakei* strains, while a high number of *L. curvatus* (26 out of 40 strains) accumulated these compounds. As already evidenced for antibiotic resistance, also the decarboxylase activity was strongly linked to the geographic origin of the isolates. The highest number of BA producing strains have been isolated from Spanish products, indicating an effect of raw materials, environmental conditions, and processes in exerting a selective pressure on microbial communities and their metabolisms (Table 4).

Table 5 BAs production by L. curvatus strains sorted by origin. *: the presence of (S) or (R) indicates an antibiotic-sensitive or antibiotic-resistant strain, respectively.

Countries of Origin	***L. Curvatus* Aminobiogenic Strains**	**Tyramine**	**Histamine**	**Putrescine**	**Cadaverine**
Italy	*L. curvatus* IAL6 (S) *	+	−	−	−
Spain	*L. curvatus* ESB8 (S)	−	−	+	−
	L. curvatus ECB11 (S)	+	−	−	−
	L. curvatus ECB12 (S)	+	−	−	−
	L. curvatus ECO46 (S)	+	−	−	−
	L. curvatus ESO6 (R)	−	−	−	−
	L. curvatus ESO13 (R)	+	−	−	−
	L. curvatus ESO14 (R)	+	−	+	−
	L. curvatus ESO16 (R)	+	−	−	−
	L. curvatus ESO19 (R)	−	−	−	−
	L. curvatus ESO25 (R)	−	−	−	−
	L. curvatus ESO52 (R)	−	−	−	−
	L. curvatus ESO59 (S)	+	−	+	−
	L. curvatus ESO61 (R)	−	−	+	−
	L. curvatus ECE16 (R)	+	−	+	−
	L. curvatus ECE25 (R)	−	−	−	−
	L. curvatus ECE27 (R)	−	+	−	−
	L. curvatus ECE32 (S)	+	−	−	−
	L. curvatus ECE35 (S)	+	−	−	−
	L. curvatus ECE37 (S)	+	−	−	−
	L. curvatus ECE40 (R)	−	−	−	−
	L. curvatus ECE42 (S)	+	−	−	−
	L. curvatus ECE46 (S)	+	−	+	−
	L. curvatus ECE51 (S)	−	−	+	−
	L. curvatus ECE52 (S)	+	−	−	−
	L. curvatus ECE53 (R)	−	−	−	−

	L. curvatus ECE54 (S)	+	−	−	−
	L. curvatus ECE57 (S)	−	−	+	−
	L. curvatus ESE3 (S)	−	−	+	−
	L. curvatus ESE19 (S)	−	−	+	−
	L. curvatus ESA38 (S)	+	−	−	−
	L. curvatus ESA53 (S)	+	−	−	−
Croatia	*L. curvatus* HZK3 (R) *L. curvatus* HZK32 (R)	+	−	−	−
		−	−	−	−
	L. curvatus HZK43 (R)	−	−	−	−
	L. curvatus HZK49 (R)	−	−	−	−
	L. curvatus HNS1 (R)	−	−	−	−
	L. curvatus HNS18 (R)	−	−	−	−
	L. curvatus HNS20 (R)	−	−	−	−
	L. curvatus HNS55 (S)	−	−	−	−
Total strains	40	19	1	10	0

Among the 40 *L. curvatus* strains, 26 were decarboxylase-positive and namely 19 produced tyramine, 10 putrescine and 1 histamine, while 4 strains were able to accumulate both tyramine and putrescine (Table 4). Seven out of 26 aminobiogenic strains presented one or more antibiotic resistances, showing different traits that are related to their safety features. Apart from enterococci, *L. curvatus* is considered the main tyramine producer among LAB in fermented sausages [20,50], while *L. sakei* is usually described as non-aminobiogenic. The decarboxylase potential has been demonstrated to be strain dependent [51]. Moreover, Ladero et al. (2015) described the capability of *L. curvatus* strains to produce both tyramine and putrescine. The latter is mainly accumulated in LAB though agmatine deiminase (AgDI) pathway, rather than ornithine decarboxylase (ODC), common in Gram negative bacteria [52].

The spontaneously fermented sausages used as source of isolation of *L. curvatus* strains presented a BA concentration ranging from about 100 mg/kg to more than 1000 mg/kg, including tyramine, putrescine and cadaverine. Interestingly, in the samples where *L. curvatus* CE-27 has been found, histamine was present at a concentration of 170 mg/kg [28].

5. CONCLUSION

Fermented sausages are produced all over Europe, with a wide diversity of manufacturing techniques and organoleptic properties between different countries and even between different regions within the same country. The 15 naturally fermented MC sausages with peculiar characteristics in terms of manufacturing and ripening conditions [28], demonstrated to be a good source of interesting autochthonous *Lactobacillaceae* to be studied for their potential technological applications in the food industry. At species level, the identified biotypes did not show a consistent biodiversity, with only *L. sakei*, dominating over the rest of the species (59.6% of isolates), *L. curvatus* present in lower proportion (26.5%) and few isolates identified as *C. alimentarius* (13.9%). Anyway, results obtained are in accordance with those described in previous studies on similar types of fermented sausages [53–56]. A more consistent biodiversity could be described in terms of strain ecology, with the Spanish sausages being the richest for the number of biotypes, while Italian and Slovenian samples showed only a low percentage of strains, belonging the majority to *L. sakei* species.

The evaluation of the safety profile of these Mediterranean products resulted in a high incidence of *L. sakei* (50%) and *L. curvatus* (45%) resistant to antibiotics; in addition, the safety assessment allowed to define a geographical clustering of resistant biotypes: strains isolated from Italian and Slovenian natural fermented sausages showed no incidence of antibiotic resistance and a negligible production of BA; on the contrary, the highest number of antibiotic-resistant isolates were detected in Spanish and Croatian salami, with a high prevalence of MDR (Multi Drug Resistant) bacteria; in particular, Streptomycin, Tetracycline, Gentamycin and Kanamycin resistances were the most observed. We found one *L. sakei* and one *L. curvatus*, which carried five resistances to antibiotics, respectively in the ECE and HZK sausages. This aspect arises a global concern linked to the safety of ready-to-eat fermented meat products; the previous large use of antibiotics in the pig production chain has led to a change in the pig microbiome and consequently in the diffusion of antibiotic resistant genes (ARG) in the meat environment [57]. The application of good manufacturing practices in the pork industry can help to control antibiotic resistant pathogen or spoilage species, but when are technological species, such as LAB, to harbor resistant genes, this can represent a difficult risk to be monitored for the consumer safety.

Finally, amino biogenic potential seemed to be species-related; in fact, no BA producers were detected among the *L. sakei* analysed strains, while a high number of producer strains was found among *L. curvatus*. After the safety assessment, a total of 45 *Lactobacillaceae* strains (44 *L. sakei* and 1 *L. curvatus* respectively) were classified to be safe, having no resistances and amino biogenic capacity. *L. sakei* demonstrated to be the most abundant species present in naturally fermented MC sausages but also the species with the best safety traits. These results could be the starting point for improved knowledge regarding the study of the technological attributes and bioprotective activity of these strains. The main aim will be to select natural starters with added value to be employed in the fresh and fermented meat productions.

6. AUTHOR CONTRIBUTION

Conceptualization, D.B.; methodology, D.B., G.T., C.M., F.G. and V.Š.; software, G.M. and M.V.B.D.; validation, D.B., G.T. and F.G.; formal analysis, G.M., F.B., M.V.B.D. and S.L.; investigation, D.B., G.M., F.B., M.V.B.D., S.L. and G.T.; resources, D.B., C.M., V.Š., F.G. and G.T.; data curation, D.B., G.M., C.M. and G.T.; writing—original draft preparation, D.B., G.M. and M.V.B.D.; writing—review and editing, D.B., G.M., M.V.B.D., C.M., F.G., V.Š. and G.T.; visualization, D.B., F.G., G.T. and C.M.; supervision, D.B., G.T. and F.G.; project administration, D.B., F.G. and G.T.; funding acquisition, D.B., F.G., V.Š. and G.T. All authors have read and agreed to the published version of the manuscript.

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8. SUPPLEMENTARY MATERIALS

Type of Sausages	Sample name	Strain	Species	Number of isolates	Absolute frequency	Relative frequency %	MIC ANTIBIOTICS								ATB genes PCR Detection							Biogenic amines production			
							Aminoglycosides			Tetracyclines	Macrolides		Chloramphenicol	Penicillins	ATB genes										
							Gentamycin	Kanamycin	Streptomyc	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	*tet* M	*tet* S	*tet* L	*tet* W	*erm* A	*erm* B	*erm* C	Tir	His	Put	Cad
Salame Fabriano - producer 1 (Marche Region)	1M	1M-8	*L. sakei*	58	20	34,48	S	S	S	S	S	S	S	S								-	-	-	-
		1M-24	*L. sakei*		24	41,38	S	S	S	S	S	S	S	S								-	-	-	-
		1M-51	*L. sakei*		14	24,14	S	S	S	S	S	S	S	S								-	-	-	-
Salame Fabriano - producer 2 (Marche Region)	2M	2M-7	*L. sakei*	48	18	37,5	S	S	S	S	S	S	S	S								-	-	-	-
		2M-9	*L. sakei*		30	62,5	S	S	S	S	S	S	S	S								-	-	-	-
Salame Alfianello (Brescia, Lombardy)	IAL	IAL-6	*L. curvatus*	67	6	8,96	S	S	S	S	S	S	S	S								+	-	-	-
		IAL-8	*L. sakei*		12	17,91	S	S	S	S	S	S	S	S								-	-	-	-
		IAL-18	*L. sakei*		20	29,85	S	S	S	S	S	S	S	S								-	-	-	-
		IAL-38	*L. sakei*		29	43,28	S	S	S	S	S	S	S	S								-	-	-	-
Traditional smoked Salami with nitrates	SN	SN-4	*L. sakei*	70	4	5,71	S	S	S	S	S	S	S	S								-	-	-	-
		SN-34	*L. sakei*		30	42,86	S	S	S	S	S	S	S	S								-	-	-	-
		SN-58	*L. sakei*		24	34,29	S	S	S	S	S	S	S	S								-	-	-	-
		SN-63	*L. sakei*		5	7,14	S	S	S	S	S	S	S	S								-	-	-	-
		SN-70	*L. sakei*		7	10,00	S	S	S	S	S	S	S	S								-	-	-	-
Traditional smoked Salami without nitrates	SWO	SWO-10	*L. sakei*	70	10	14,29	S	S	S	S	S	S	S	S								-	-	-	-
		SWO-18	*L. sakei*		8	11,43	S	S	S	S	S	S	S	S								-	-	-	-
		SWO-29	*L. sakei*		11	15,71	S	S	S	S	S	S	S	S								-	-	-	-
		SWO-48	*L. sakei*		19	27,14	S	S	S	S	S	S	S	S								-	-	-	-
		SWO-60	*L. sakei*		12	17,14	S	S	S	S	S	S	S	S								-	-	-	-
		SWO-61	*L. sakei*		10	14,29	S	S	S	S	S	S	S	S								-	-	-	-
Salchichón Bérchules	ESB	ESB-2	*L. sakei*	69	2	2,90	S	S	S	S	S	S	S	S								-	-	-	-
		ESB-7	*L. sakei*		5	7,25	S	S	S	S	S	S	S	S								-	-	-	-
		ESB-8	*L. curvatus*		1	1,45	S	S	S	S	S	S	S	S								-	-	+	-
		ESB-14	*L. sakei*		6	8,70	S	S	S	S	S	S	S	S								-	-	-	-
		ESB-24	*L. sakei*		10	14,49	S	S	S	S	S	S	S	S								-	-	-	-
		ESB-26	*L. sakei*		2	2,90	S	S	R	S	S	S	S	S								-	-	-	-
		ESB-34	*L. sakei*		8	11,59	S	S	R	S	S	S	S	S								-	-	-	-
		ESB-53	*L. sakei*		17	24,64	S	S	S	S	S	S	S	S								-	-	-	-
		ESB-57	*L. sakei*		4	5,80	S	S	S	R	S	S	S	S	x	x						-	-	-	-
		ESB-60	*L. sakei*		5	7,25	S	S	S	S	S	S	S	S								-	-	-	-
		ESB-67	*L. sakei*		9	13,04	S	S	S	S	S	S	S	S								-	-	-	-
Chorizo Bérchules	ECB	ECB-1	*C. alimentarius*	48	2	4,17																			
		ECB-3	*C. alimentarius*		3	6,25																			
		ECB-6	*C. alimentarius*		2	4,17																			
		ECB-8	*C. alimentarius*		2	4,17																			
		ECB-10	*C. alimentarius*		1	2,08																			
		ECB-11	*L. curvatus*		1	2,08	S	S	S	S	S	S	S	S								+	-	-	-
		ECB-12	*L. curvatus*		1	2,08	S	S	S	S	S	S	S	S								+	-	-	-
		ECB-13	*C. alimentarius*		3	6,25																			
		ECB-16	*C. alimentarius*		2	4,17																			
		ECB-17	*C. alimentarius*		2	4,17																			
		ECB-22	*C. alimentarius*		3	6,25																			
		ECB-31	*C. alimentarius*		9	18,75																			
		ECB-36	*C. alimentarius*		5	10,42																			
		ECB-39	*C. alimentarius*		3	6,25																			
		ECB-41	*C. alimentarius*		2	4,17																			
		ECB-43	*C. alimentarius*		7	14,58																			
Chorizo Olvera	ECO	ECO-12	*C. alimentarius*	52	12	23,08																			
		ECO-14	*C. alimentarius*		2	3,85																			
		ECO-19	*L. sakei*		5	9,62	R	S	S	R	S	S	S	S	x							-	-	-	-
		ECO-24	*C. alimentarius*		5	9,62																			
		ECO-38	*L. sakei*		14	26,92	S	S	S	S	S	S	S	S								+	-	-	-
		ECO-46	*L. curvatus*		8	15,38	S	S	S	S	S	S	S	S								+	-	-	-
		ECO-50	*C. alimentarius*		6	11,54																			
Salchichón Olvera	ESO	ESO-6	*L. curvatus*	70	3	4,29	S	S	S	R	S	S	S	S								-	-	-	-
		ESO-3	*C. alimentarius*		3	4,29																			
		ESO-8	*L. sakei*		2	2,86	S	S	S	S	S	S	S	S								-	-	-	-
		ESO-10	*L. sakei*		2	2,86	S	S	S	S	S	S	S	S								-	-	-	-
		ESO-13	*L. curvatus*		3	4,29	S	S	S	R	S	S	S	S								+	-	-	-
		ESO-14	*L. curvatus*		1	1,43	S	S	S	R	S	S	S	S								+	-	+	-
		ESO-16	*L. curvatus*		2	2,86	S	S	R	S	S	S	S	S								+	-	-	-
		ESO-19	*L. curvatus*		3	4,29	S	S	R	S	S	S	S	R									-	-	-
		ESO-23	*L. sakei*		4	5,71	S	S	S	S	S	S	S	S								-	-	-	-
		ESO-24	*L. sakei*		1	1,43	S	S	S	R	S	S	S	S								-	-	-	-
		ESO-25	*L. curvatus*		1	1,43	S	S	S	R	S	S	S	S								-	-	-	-
		ESO-38	*L. sakei*		13	18,57	S	S	S	S	S	S	S	S								-	-	-	-
		ESO-47	*L. sakei*		9	12,86	S	S	S	S	S	S	S	S								-	-	-	-
		ESO-52	*L. curvatus*		5	7,14	S	S	R	R	R	S	S	S						x		-	-	-	-
		ESO-53	*L. sakei*		1	1,43	S	S	R	S	S	S	S	S								-	-	-	-
		ESO-56	*L. sakei*		3	4,29	S	S	R	S	S	S	S	S								-	-	-	-
		ESO-59	*L. curvatus*		3	4,29	S	S	S	S	S	S	S	S								+	-	+	-
		ESO-61	*L. curvatus*		6	8,57	S	S	R	S	S	S	S	S								-	-	+	-
		ESO-65	*L. sakei*		5	7,14	S	S	S	S	S	S	S	S								-	-	-	-
Chorizo Écija	ECE	ECE-2	*L. sakei*	69	2	2,90	S	S	S	S	S	S	S	S								-	-	-	-
		ECE-3	*L. sakei*		1	1,45	S	S	R	S	S	S	S	S								-	-	-	-
		ECE-4	*L. sakei*		1	1,45	S	S	R	S	S	S	S	S								-	-	-	-
		ECE-5	*L. sakei*		1	1,45	R	R	R	S	S	R	R	S								-	-	-	-
		ECE-6	*L. sakei*		1	1,45	S	S	S	R	S	S	S	S								-	-	-	-
		ECE-10	*L. sakei*		5	7,25	R	S	R	S	S	S	S	S								-	-	-	-
		ECE-15	*L. sakei*		4	5,80	R	S	R	S	S	S	S	S								-	-	-	-
		ECE-16	*L. curvatus*		4	5,80	S	S	S	S	S	S	R	S								+	-	+	-
		ECE-25	*L. curvatus*		9	13,04	S	S	S	S	S	S	R	S								-	-	-	-
		ECE-27	*L. curvatus*		2	2,90	S	S	S	R	S	S	S	S								-	+	-	-
		ECE-32	*L. curvatus*		5	7,25	S	S	S	S	S	S	S	S								+	-	-	-
		ECE-35	*L. curvatus*		3	4,35	S	S	S	S	S	S	S	S								+	-	-	-
		ECE-37	*L. curvatus*		2	2,90	S	S	S	S	S	S	S	S								+	-	-	-
		ECE-40	*L. curvatus*		3	4,35	S	S	R	S	S	S	S	S								-	-	-	-
		ECE-42	*L. curvatus*		2	2,90	S	S	S	S	S	S	S	S								+	-	-	-
		ECE-46	*L. curvatus*		4	5,80	S	S	S	S	S	S	S	S								+	-	+	-
		ECE-49	*C. alimentarius*		3	4,35																			
		ECE-51	*L. curvatus*		2	2,90	S	S	S	S	S	S	S	S								-	-	+	-
		ECE-52	*L. curvatus*		1	1,45	S	S	S	S	S	S	S	S								+	-	-	-
		ECE-53	*L. curvatus*		1	1,45	S	S	S	S	S	S	R	S								-	-	-	-
		ECE-54	*L. curvatus*		1	1,45	S	S	S	S	S	S	S	S								+	-	-	-
		ECE-57	*L. curvatus*		12	17,39	S	S	S	S	S	S	S	S								-	-	+	-
Salchichón Écija	ESE	ESE-3	*L. curvatus*	69	3	4,35	S	S	S	S	S	S	S	S								-	-	+	-
		ESE-14	*C. alimentarius*		11	15,94																			
		ESE-19	*L. curvatus*		5	7,25	S	S	S	S	S	S	S	S								-	+	+	-
		ESE-24	*L. sakei*		5	7,25	R	S	R	S	S	S	S	S								-	-	-	-
		ESE-30	*L. sakei*		6	8,70	S	S	S	S	S	S	S	S								-	-	-	-
		ESE-32	*L. sakei*		2	2,90	R	S	S	S	S	S	S	S								-	-	-	-
		ESE-41	*L. sakei*		9	13,04	S	S	S	S	S	S	S	S								-	-	-	-
		ESE-67	*L. sakei*		28	40,58	S	S	S	S	S	S	S	S								-	-	-	-
Salchichón Alhendín	ESA	ESA-4	*L. sakei*	67	4	5,97	R	S	S	S	S	S	S	S								-	-	-	-
		ESA-21	*L. sakei*		17	25,37	S	S	S	S	S	S	S	S								-	-	-	-
		ESA-25	*L. sakei*		4	5,97	S	S	R	S	S	S	S	S								-	-	-	-
		ESA-37	*L. sakei*		12	17,91	S	S	R	S	S	S	S	S								-	-	-	-
		ESA-38	*L. curvatus*		1	1,49	S	S	S	S	S	S	S	S								+	-	-	-
		ESA-49	*L. sakei*		11	16,42	S	S	S	S	S	S	S	S								-	-	-	-
		ESA-53	*L. curvatus*		4	5,97	S	S	S	S	S	S	S	S								+	-	-	-
		ESA-56	*L. sakei*		3	4,48	S	S	R	S	S	S	S	S								-	-	-	-
		ESA-59	*L. sakei*		3	4,48	S	S	R	S	S	S	S	S								-	-	-	-
		ESA-62	*L. sakei*		3	4,48	R	R	R	S	S	S	S	S								-	-	-	-
		ESA-63	*L. sakei*		5	7,46	R	R	R	S	S	S	S	S								-	-	-	-
Salami Zminjska Klobasica	HZK	HZK-3	*L. curvatus*	53	3	5,66	R	R	R	S	S	S	S	S								+	-	-	-
		HZK-5	*L. sakei*		2	3,77	S	S	R	S	S	S	S	S								-	-	-	-
		HZK-6	*L. sakei*		1	1,89	S	S	S	R	S	S	S	S								-	-	-	-
		HZK-10	*L. sakei*		6	11,32	S	S	S	R	S	S	S	S								-	-	-	-
		HZK-11	*L. sakei*		1	1,89	S	S	S	R	S	S	S	S								-	-	-	-
		HZK-12	*L. sakei*		1	1,89	S	S	S	R	S	S	S	S								-	-	-	-
		HZK-15	*L. sakei*		3	5,66	S	S	R	S	S	S	S	S								-	-	-	-
		HZK-18	*L. sakei*		1	1,89	R	S	R	S	S	S	S	S								-	-	-	-
		HZK-19	*L. sakei*		1	1,89	S	R	R	S	S	R	S	S								-	-	-	-
		HZK-23	*L. sakei*		4	7,55	R	S	S	S	S	S	S	S								-	-	-	-
		HZK-26	*L. sakei*		3	5,66	S	R	S	S	S	S	R	S								-	-	-	-
		HZK-30	*L. sakei*		4	7,55	S	R	S	S	S	R	S	S								-	-	-	-
		HZK-32	*L. curvatus*		2	3,77	R	R	R	S	S	S	S	S								-	-	-	-
		HZK-36	*L. sakei*		4	7,55	R	R	R	S	S	S	S	S								-	-	-	-
		HZK-39	*L. sakei*		3	5,66	S	S	S	S	S	S	S	S								-	-	-	-
		HZK-42	*L. sakei*		3	5,66	S	S	S	S	S	S	S	S								-	-	-	-
		HZK-43	*L. curvatus*		1	1,89	R	S	R	S	S	R	S	S								-	-	-	-
		HZK-47	*L. sakei*		4	7,55	S	S	S	R	S	S	S	S								-	-	-	-
		HZK-49	*L. curvatus*		2	3,77	R	R	R	S	R	R	S	S								-	-	-	-
		HZK-50	*L. sakei*		1	1,89	S	S	S	S	S	S	S	S						x		-	-	-	-
		HZK-53	*L. sakei*		3	5,66	R	S	R	S	S	S	S	S								-	-	-	-
Traditional smoked Salami	HS	HS-1	*L. sakei*	49	2	4,08	S	R	R	S	S	S	S	S								-	-	-	-
		HS-2	*L. sakei*		6	12,24	S	R	R	S	S	S	S	S								-	-	-	-
		HS-8	*L. sakei*		4	8,16	S	S	S	R	S	S	S	S								-	-	-	-
		HS-13	*L. sakei*		15	30,61	S	R	R	R	S	S	S	S								-	-	-	-
		HS-42	*L. sakei*		22	44,90	S	R	S	S	S	S	R	S								-	-	-	-
Traditional unsmoked Salami	HNS	HNS-1	*L. curvatus*	55	1	1,82	S	S	R	S	S	S	S	S								-	-	-	-
		HNS-3	*L. sakei*		2	3,64	S	S	S	R	S	S	S	S								-	-	-	-
		HNS-7	*L. sakei*		4	7,27	S	S	S	R	S	S	S	S	x	x						-	-	-	-
		HNS-12	*L. sakei*		5	9,09	S	S	S	R	S	S	S	S		x						-	-	-	-
		HNS-16	*L. sakei*		4	7,27	S	S	S	R	S	S	S	S								-	-	-	-
		HNS-18	*L. curvatus*		2	3,64	R	S	R	S	S	S	S	R								-	-	-	-
		HNS-20	*L. curvatus*		2	3,64	S	S	S	R	S	R	R	S								-	-	-	-
		HNS-21	*L. sakei*		1	1,82	S	S	S	S	S	S	S	S								-	-	-	-
		HNS-28	*L. sakei*		7	12,73	S	S	S	S	S	S	S	S								-	-	-	-
		HNS-48	*L. sakei*		20	36,36	S	S	S	S	S	S	S	S								-	-	-	-
		HNS-55	*L. curvatus*		7	12,73	S	S	S	S	S	S	S	S								-	-	-	-

Table S1*. List of strains used in this study with respective origin, MIC, ATB PCR detection and biogenic amines prediction.*

CHAPTER 7

Technological traits and mitigation activity of autochthonous lactic acid bacteria from Mediterranean fermented meat-products

Technological traits and mitigation activity of autochthonous lactic acid bacteria from Mediterranean fermented meat-products

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Keywords: autochthonous lactic acid bacteria, fermented sausages, bacteriocins, bio-protection, growth performances

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1. ABSTRACT

The production of safe and standardized fermented sausages with typical characteristics linked to traditional origin is highly desirable. The use of autochthonous starter cultures that provide peculiar flavor, texture and color to the fermented products, while maintaining the meat-product safe can be a feasible strategy for producers. In this study, 45 strains of *Latilactobacillus sakei* and 1 *Latilactobacillus curvatus* isolated from natural Mediterranean fermented sausages, were screened as potential protective cultures for their use in the fermented sausage industry. Technological properties, inhibitory activity towards *Escherichia coli* and *Listeria innocua* and the presence of genes coding for bacteriocins, were investigated. All tested strains showed an antagonistic effect by growing, while inhibiting the growth of target harmful microorganisms, in a strain-specific manner. At least one bacteriocin encoding genes was present in 25 strains, mainly sakacin X and sakacin P. The technological performances of the strains highlighted a great variability in the behavior, confirming the phenotypic diversity already reported for LAB species highly adapted to meat environment. Results highlight the potentiality of these strains to be used as protective starters in fermented meat products to improve food quality and microbiological safety, as well as giving peculiar characteristics to the final product.

2. INTRODUCTION

Food-borne diseases are a major cause of morbidity and mortality worldwide, causing up to 600 million cases of foodborne illness and 420,000 deaths per year (Lee & Yoon, 2021). This represents a substantial health burden for governments, which incur in greater expenditure to healthcare and medical expenses (Erdoğmuş et al., 2021; Faour-Klingbeil & Todd, 2020; Lee & Yoon, 2021). The ability of foodborne pathogens to grow, create biofilms and toxin production represent dangerous aspects connected to pathogenicity, outbreaks and affect consumers health (Janež et al., 2021; Kim & Kim, 2012). Consequently, there has been a growing demand from consumers in recent years for healthy and safe food (de Andrade et al., 2019; Gressier et al., 2020).

Manufacturing of standardized and safe food products, but still characterized by traditional and regional organoleptic and nutritional properties, represents a main issue for food companies, which aim to find a strategy to meet all the market requests (Gizaw, 2019). In this perspective, the use of autochthonous starter cultures can be a useful tool to achieve the production of safe and high quality traditional foods (Lorenzo et al., 2017). Moreover, indigenous starter cultures are known to often improve the organoleptic features of fermented products such as taste, texture and color (dos Santos Cruxen et al., 2019; Terzić-Vidojević et al., 2020).

Among processed foods, meat and meat products pose a significant challenge to food companies in ensuring safety of final products (Belloso Daza et al., 2022; Devleesschauwer et al., 2019). In fact, microbial contaminations of fresh and processed meats by various pathogenic and spoilage microorganisms has become a major issue for consumers health (Fegan & Jenson, 2018; Huffaker & Hartmann, 2021). In the last decades, these issues moved companies and researchers to broaden their

knowledge about new control measures, such as bioprotectants and protective food cultures (Falardeau et al., 2021; Sameli & Samelis, 2022). Physical methods alone are frequently not sufficient to ensure the production of safe and reliable foods (Lahiri et al., 2022). A possible strategy is the incorporation of lactic acid bacteria (LAB), with the capacity to produce antimicrobial compounds, during the manufacturing process. Given their GRAS (generally recognized as safe) status, LAB are extensively employed in the meat industry as starter cultures to facilitate fermentation and/or as biocontrol agents, thereby addressing these concerns effectively (Patricia Castellano et al., 2017; Mathur et al., 2020; Raman et al., 2022).

LAB antimicrobial activity against foodborne pathogens and spoilage agents can be exerted by different metabolites produced during their growth such as organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins (Patricia Castellano et al., 2017; Chen et al., 2021; da Costa et al., 2019). Their inhibitory activity has been extensively studied in food matrices to evaluate the effect against the growth of pathogenic or spoilage microorganisms (Baillo et al., 2023; Ben Said et al., 2019; Danielski et al., 2022; Segli et al., 2021; Todorov et al., 2017; Xu et al., 2021). Several studies documented that a wide range of LAB strains, which include *Latilactobacillus sakei*, *Lactiplantibacillus plantarum*, *Ligilactobacillus animalis* and *Latilactobacillus curvatus*, can be used as effective bioprotective microorganisms in meat and meat products (Castellano et al., 2012; Jones et al., 2010; Li et al., 2016; Tirloni et al., 2014).

Food industries are continuously searching for autochthonous indigenous bacteria (LAB and Gram-positive catalase cocci), endowed with technological and antimicrobial features, that can be potentially used as new starter and, at the same time, as protective cultures in meat products (dos Santos Cruxen et al., 2019)

In the selection of potential LAB starter cultures, proper growth performances at different temperatures, even in the presence of high concentrations of NaCl and the consequent rapid pH drop in the meat matrix, are the most important technological characteristics (Nikodinoska et al., 2023). In addition, the contribution of candidate strains to the aroma profile formation is relevant for the sensorial acceptability and product recognizability (Carballo, 2021; Montanari et al., 2018).

In this study 45 *L. sakei* and 1 *L. curvatus* strains, isolated from spontaneously fermented sausages produced in the Mediterranean area and previously screened regarding their safety aspects (Barbieri et al., 2021; Bassi et al., 2022), were characterized for their ability to inhibit pathogenic microorganisms *in vitro* and in meat models. Moreover, the presence of genes related to the production of bacteriocins was assessed. The most promising strains were then analyzed for their technological properties. Growth kinetics at different salt concentrations and different temperatures were studied with the aim to exploit the microbial biodiversity of LAB populations in European fermented sausages and select new autochthonous starter cultures for traditional products manufacture.

3. MATERIALS AND METHODS

3.1. Strains and growth conditions

The 45 strains of *Latilactobacillus sakei* and 1 *Latilactobacillus curvatus* considered in this study are reported in Table 1, in relation to their isolation source (Bassi et al., 2022). Selected microorganisms were cultivated in MRS broth (Oxoid, Italy) for 48 h at 37°C under anaerobic conditions. *Escherichia coli* ATCC 25922 and *Listeria innocua* UC8409, used to test the LAB inhibitory activity, were grown in BHI broth (Oxoid, Italy) overnight at 37°C. After incubation, samples were stocked at -40°C in MRS broth and BHI broth respectively, containing 20% glycerol (Carlo Erba, Italy) until the beginning of experiments.

Table 6 Strains of Latilactobacillus isolated from different naturally fermented Mediterranean sausages.

Isolation source (type of sausages and Countries)	**Strains**	**Species**
Salame Fabriano - producer 1 (Italy)	1M8	*L. sakei*
	1M24*	*L. sakei*
	1M51	*L. sakei*
Salame Fabriano - producer 2 (Italy)	2M7*	*L. sakei*
	2M9*	*L. sakei*
Salame Alfianello (Italy)	IAL8*	*L. sakei*
	IAL18	*L. sakei*
	IAL38	*L. sakei*
Traditional smoked salami with nitrates (Slovenia)	SN4	*L. sakei*
	SN34*	*L. sakei*
	SN58*	*L. sakei*
	SN63	*L. sakei*
	SN70	*L. sakei*
Traditional smoked salami without nitrates (Slovenia)	SWO10*	*L. sakei*
	SWO18	*L. sakei*
	SWO29	*L. sakei*
	SWO48	*L. sakei*
	SWO60	*L. sakei*
	SWO61*	*L. sakei*
Salchichón Alhendín (Spain)	ESA21	*L. sakei*
	ESA49	*L. sakei*
Salchichón Bérchules (Spain)	ESB2*	*L. sakei*
	ESB7	*L. sakei*
	ESB14*	*L. sakei*
	ESB24	*L. sakei*
	ESB53	*L. sakei*
	ESB60	*L. sakei*
	ESB67	*L. sakei*
Salchichón Écija (Spain)	ESE30*	*L. sakei*
	ESE41	*L. sakei*
	ESE67	*L. sakei*
Salchichón Olvera (Spain)	ESO8*	*L. sakei*
	ESO10	*L. sakei*
	ESO23*	*L. sakei*
	ESO38	*L. sakei*
	ESO47	*L. sakei*
	ESO65*	*L. sakei*
Chorizo Écija (Spain)	ECE2*	*L. sakei*
Chorizo Olvera (Spain)	ECO38*	*L. sakei*
Traditional unsmoked salami (Croatia)	HNS21	*L. sakei*
	HNS28	*L. sakei*
	HNS48*	*L. sakei*
	HNS55*	*L. curvatus*
Salami Zminjska Klobasica (Croatia)	HZK39*	*L. sakei*
	HZK42*	*L. sakei*
	HZK50	*L. sakei*

*strains selected for further technological characterization.

3.2. Inhibitory activity against E. coli ATCC25922 and List. innocua UC8409 with agar overlay assay

The agar overlay method was used to test the inhibitory capacity of the LAB strains against *E. coli* ATCC 25922 and *List. innocua* UC8409, selected as the non-pathogenic counterpart of Shiga toxin producing *Escherichia coli* (STEC) and *List. monocytogenes*, as previously described by Mendling and colleagues with some modifications (Mendling, 2017). Briefly, 10 µl of an overnight culture of the LAB microorganism to be tested, were spotted on MRS agar plates and incubated for 48 h at 30°C under anaerobic conditions. After, each MRS plate was overlayed with 10ml of BHI with the addition of 0.8% bacteriological agar (Oxoid) previously inoculated with 7 log CFU/ml of *E. coli* ATCC 25922 or *List. innocua* UC8409. After solidification of the overlayed agar medium, the plates were incubated at 30°C for 48 h. The analysis was performed in triplicate. Once the incubation time has expired the diameter of the inhibition halos was measured and the results were analyzed using the following scale:

- (+++): diameter > 4 cm;
- (++): diameter 2-4 cm;
- (+): diameter 0.5-2 cm;
- (-): no halo.

3.3. Inhibitory activity of *Latilactobacillus* against *E. coli* ATCC25922 and *List. innocua* UC8409 in sausage meat models

The fermented sausage (salami) meat model composition was reported in table 2. Salami meat was finely minced in sterile conditions; then, 60 g were taken, supplemented with 12 ml of sterile water and pasteurized at 65°C for 30 min. After, 180 ml of a 2% water-agar solution were added. The resulting mixture was mixed for 3 min and then filtered to remove particulate material. After filtration, 6.3 ml of 20% glucose solution was added in order to obtain a final concentration of 0.5%. Finally 2.5 ml of 1% solution of 2,3,5-triphenyltetrazolium chloride (MERC) was added to allow the enumeration of colonies (Beloti et al., 1999).

Agar-salami medium was cooled at 50°C and then poured on sterile 25-well plates (Thermo Fisher Scientific). Then, 30 µl of a 6 log CFU/ml concentrated culture of each *Latilactobacillus* (Table 1) were spotted separately in each well. Subsequently the agar-salami medium was poured, and the inoculum was homogenized with a sterile loop. After solidification of the media, overnight cultures of *List. innocua* UC8409 with a microbial cell load of $8x10^8$ CFU/ml and *E. coli* ATCC 25922 with a concentration of $1x10^9$ CFU/ml, were serially diluted 7 times with saline solution. Then, 30 µl of each obtained diluted microorganism was spotted on the surface of 1 agar-salami well, previously inoculated with *L. sakei* or *L. curvatus*. As a positive control 30 µl of each dilution of *List. innocua* and *E. coli* were inoculated on the surface of agar-salami without the addition of any *Latilactobacillus*. The plates, containing 8 wells per sample, were first incubated at 37°C for 24 h under anaerobic conditions

followed by other 24 h at room temperature in aerobic conditions. Same conditions were maintained also for positive controls, without adding any *Latilactobacillus* strain. The results obtained were expressed as the logarithmic reduction of growth when compared to the positive control. pH values for each tested strain were measured and was expressed as the mean of eight growing wells.

Table 7 Fermented sausage meat model composition.

Ingredient	Amount	Final concentration
Meat	60 g	
H_20	12 ml	
Bacteriological agar (Oxoid)	180 ml	
Glucose 20% (Carlo Erba)	6.3 ml	0.5%
2,3,5-triphenyltetrazolium chloride 1% (Sigma)	2.5 ml	0.01%
Final volume	250 ml	

3.4. Gene-specific PCR for the detection of genes coding for bacteriocins

The presence of genes related to the production of bacteriocins was investigated for the 45 strains of *L. sakei* and one *L. curvatus*. DNA was extracted from LAB strains using the NucleoSpin® Tissue (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturers instructions in order to perform the gene-specific PCR test for the detection of genes encoding bacteriocins. Extracted DNA was PCR processed according to previous protocols (Barbosa et al., 2014; Dortu et al., 2008; Fontana et al., 2015). The presence of genes coding for Curvacin A (*curA*), Sakacin P (*sakP*), Sakacin Q (*sakQ*), Sakacin G (*sakG*), Sakacin Tα (*sakTa*), Sakacin Tβ (*sakTβ*), Sakacin X (*sakX*) was detected. PCR was performed on a total volume of 25 µl containing 12.5 µl of PCR Master Mix, 2X (Promega, Germany), 1.25 µl of each primer at concentration of 10 µM, 8 µl of nuclease free water (Promega, Germany) and 2 µl of DNA. We used two different PCR profiles to detect genes for different 21 bacteriocins; primers and PCR conditions are described in Table 3. The amplified products were separated in 1.5% agarose gel and visualized by Sybr-Safe staining. Positive controls (2 µl of a reference strain) were also included in the amplification runs. DNA from *L. curvatus* M05 was used as positive control of *SakQ, SakP, SakG, SakT, SakT* e *SakX* amplification, while *L. curvatus* 705 and R212 as positive control of *CurA* detection.

Table 8 Primers and PCR profiles used for the detection of bacteriocin coding genes.

Target bacteriocin	Primer Name	Sequence (5'-3')	Size (bp)	PCR Profile	Reference
Sakacin P	SakP-F	GAA(T/A)T(A/G)(C/A)(A/C)ANCAATTA (C/T)(A/C)GGTGG	124	94°Cx5', 35 x (94°Cx30 '', 50°Cx45", 72°Cx1'), 72°Cx7'	(Dortu et al., 2008)
	SakP-R	GGCCCAGTTTGCAGCTGCAT			

Sakacin T	SakTα-F	TCGGTGGCTATACTGCTAAACA	160	94°Cx5', 35x (94°Cx 30",50°Cx45",72°Cx1'), 72°Cx7	(S.J. Macwana & Muriana, 2012)
	SakTα-R	TGTCCTAAAAATCCACCAATGC			
Sakacin T	SakTβ-F	AAGAAATGATAGAAATTTTTGGAGG	151	94°Cx5',35x (94°Cx 30",50°Cx45",72°Cx1'), 72°Cx7	
	SakTβ-R	TGTGAAATCCAATCTTGTCCTG			
Sakacin Q	SakQ-F	GAA(T/A)T(A/G)(C/A)(A/C)ANCAATTA(C/T)(A/C)GGTGG	130	94°Cx5', 35x (94°Cx30 ", 50°Cx45", 72°Cx1'), 72°Cx7'	(Dortu et al., 2008)
	SakQ-R	TACCACCAGCAGCCATTCCC			
Sakacin X	SakX-F	AGCTATGAAAGGTATTGTCGGG	156	94°Cx5', 35x (94°Cx 30",50°Cx45",72°Cx1'), 72°Cx7'	(S. Macwana & Muriana, 2012)
	SakX-R	TAAGATTTCCAGCCAGCAGC			
Sakacin G	SakG-F	GTAAAAATTATTTAACAGGAGG	492	94°Cx5', 35 x (94°Cx30 ", 50°Cx45", 72°Cx1'), 72°Cx7'	(Dortu et al., 2008)
	SakG-R	TTAGTGCTTTTTTATCTGGTA			
Curvacin A	curA-F	GTAAAAGAAITAAGTATGACA	171	94°Cx5', 35 x (94°Cx30 ", 50°Cx45", 72°Cx1'), 72°Cx7'	(Remiger et al., 1996)
	curA-R	ITACATTCCAGCTAAACCACT			

3.5. Growth performances in presence of different salt concentrations at different incubation temperatures

Based on results obtained from their antimicrobial activity, 19 *L. sakei* and 1 *L. curvatus* were chosen to perform further technological analyses (strains highlighted with * on Table 1). LAB strains were evaluated for their growth performances in MRS broth in relation of different salt concentrations (0%, 2.5% and 5% NaCl) at 20°C and at different incubation temperatures (10°C, 20°C and 30°C) in the absence of salt. They were pre-cultivated in MRS broth for 24 h at 30°C and then inoculated to a final concentration of 5 log CFU/ml into the different media for further analyses. During incubation, their growth was monitored through the variation of optical density at 600 nm, measured with an UV-VIS spectrophotometer 6705 UV-Vis (Jenway, Stone, UK).

3.6. Predictive microbiology models

Collected data were elaborated by using predictive microbiology models. In this context, Gompertz equation (1), as modified by Zwietering et al. (1990), was used to model them with Statistica 8.0 software (StatSoft Inc.) (Zwietering et al., 1990):

$$OD_{600} = A \cdot e^{-e^{((\frac{\mu max \cdot e}{A}) \cdot (\lambda - t) + 1)}} \quad (1)$$

where A represent the maximum OD_{600} value reached (OD_{600}), μ_{max} is the maximum OD_{600} increase rate in exponential phase (h^{-1}) and λ is the lag phase (h). pH in MRS broth was measured with pH-meter Basic 20 (Crison Instruments). The initial pH of the growth medium was 5.9 ± 0.07.

3.7. Statistical analysis

Parameters obtained with Gompertz equation modelling were elaborated through statistical software R (R Core Team, 2009). The analyses were performed by using "boxplot" function. These data were also analyzed through a one-way ANOVA model by addition of "lme4" (Bates et al., 2015) and "emmeans" (Lenth et al., 2018) packages in software R. All statistical differences were considered significant at a level of $P \leq 0.05$ using the Bonferroni test.

4. RESULTS AND DISCUSSION

4.1. Inhibitory activity with Agar Overlay Assay against *E. coli* ATCC 25922 and *List. innocua* UC8409

This study aims to test the ability of different LAB strains, belonging to *L. sakei* and *L. curvatus* species, isolated from spontaneously fermented sausages produced in the Mediterranean area, in inhibiting pathogens that could be considered as possible contaminants in meat products. This approach was meant to identify the most inhibiting wild strains. Antimicrobial activity of the 46 selected LAB strains (45 *L. sakei* and 1 *L. curvatus*) was evaluated against *E. coli* ATCC 25922 and *List. innocua* UC8409 as candidate microorganisms. Data reported in Table 4 show that all tested LAB inhibited the growth of *E. coli* and *List. innocua* at different levels. Inhibition of pathogens proved to be dependent on the specific tested strain. In fact, as expressed by the results of inhibition halo tests, *E. coli* was the most sensible microorganism to inhibitory effect exerted by LAB. To support this outcome, 15 strains of *L. sakei* out of 45 and the only *L. curvatus* created an inhibition zone between 2.4 and 3.2 cm. 30 out of 45 *L. sakei* tested strains generated an inhibition zone between 1.6 and 2.4 cm. On the other hand, *List. innocua* was inhibited to a minor extent with only 8 strains of *L. sakei* out of 45 able to form an inhibition zone comprised between 2.6 and 3.4 cm and 34 strains that create inhibition zones of 1.6 to 2.4 cm. The only *L. curvatus* isolated from Croatian salami Zminjska Klobasica tested shows good inhibition performances creating an inhibition zone >4 cm against both pathogens. Similarly, a total of 7 *L. sakei* strains, 6 isolated from Croatian salami Zminjska Klobasica and one present in Spanish Salchichón Alhendín,

demonstrated the highest inhibitory effect (+++) against both microorganisms. Low differences between inhibitory performance of *L. sakei* and *L. curvatus* are supported by the literature where it has been also reported that these two microorganisms, traditionally bounded to meat products (Hugas, 1998), are phylogenetic and metabolically close (Lopez-Arvizu et al., 2021).

Table 9 Inhibition halo of the 46 LAB against E. coli and List. innocua. Inhibition zones: (+++) > 4 cm; (++) 2-4 cm; (+) 0.5-2 cm; (- no halo).

Strains	**Species**	***E. coli* ATCC 25922**	***List. innocua* UC8409**
1M8	*L. sakei*	(++)	(+)
1M24	*L. sakei*	(++)	(+)
1M51	*L. sakei*	(++)	(+)
2M7	*L. sakei*	(++)	(+)
2M9	*L. sakei*	(++)	(+)
IAL8	*L. sakei*	(+)	(+)
IAL18	*L. sakei*	(+)	(+)
IAL38	*L. sakei*	(+)	(++)
SN4	*L. sakei*	(++)	(+)
SN34	*L. sakei*	(++)	(+)
SN58	*L. sakei*	(++)	(+)
SN63	*L. sakei*	(++)	(+)
SN70	*L. sakei*	(++)	(+)
SWO10	*L. sakei*	(++)	(+)
SWO18	*L. sakei*	(++)	(+)
SWO29	*L. sakei*	(++)	(+)
SWO48	*L. sakei*	(++)	(++)
SWO60	*L. sakei*	(++)	(+)
SWO61	*L. sakei*	(++)	(+)
ESA21	*L. sakei*	(++)	(+)
ESA49	*L. sakei*	(++)	(+)
ESB2	*L. sakei*	(++)	(++)
ESB7	*L. sakei*	(+++)	(+++)
ESB14	*L. sakei*	(++)	(+)
ESB24	*L. sakei*	(++)	(+)
ESB53	*L. sakei*	(++)	(+)
ESB60	*L. sakei*	(++)	(+)
ESB67	*L. sakei*	(++)	(+)
ESE30	*L. sakei*	(++)	(+)
ESE41	*L. sakei*	(++)	(+)
ESE67	*L. sakei*	(++)	(+)
ESO8	*L. sakei*	(++)	(+)
ESO10	*L. sakei*	(++)	(+)
ESO23	*L. sakei*	(++)	(++)
ESO38	*L. sakei*	(++)	(+)
ESO47	*L. sakei*	(+)	(+)
ESO65	*L. sakei*	(+)	(+)
ECE2	*L. sakei*	(+)	(+)
ECO38	*L. sakei*	(++)	(+)
HNS21	*L. sakei*	(+++)	(+++)
HNS28	*L. sakei*	(+++)	(+++)
HNS48	*L. sakei*	(+++)	(+++)
HNS55	*L. curvatus*	(+++)	(+++)
HZK39	*L. sakei*	(+++)	(+++)

HZK42	*L. sakei*	(+++)	(+++)
HZK50	*L. sakei*	(+++)	(+++)

4.2. Inhibition of *E. coli* ATCC 25922 and *List. innocua* UC8409 by LAB strains in fermented sausage model

Results of *in vitro* agar overlay assay, although promising, can only suggest the ability of viable cells to inhibit the growth of pathogenic microorganisms. For this reason, an inhibition assay on agar-salami media was assessed. This medium was prepared starting from pork meat to maintain the same nutritional and physicochemical characteristics of the real product. Meat batter purchased from a local market and used in this model was selected to allow the growth of LAB and target microorganisms to be inhibited, mimicking the processes that take place in the real product. The mitigation activity of *Latilactobacillus* strains was assessed by comparing the growth and the inhibition of the target microorganism with and without the addiction of the LAB cultures. The inhibition rate was expressed as log CFU reduction. The results reported in Table 5, indicated that all the strains were able to inhibit the growth of selected microorganisms to some extent, except for the strain IAL18 that did not cause any reduction of *List. innocua*. Data also confirmed that *E. coli* showed higher sensitivity to LAB inhibition with respect to *List. innocua*, with 12 strains of *L. sakei* able to limit the growth of *E. coli* of at least 4 log CFU in the meat model. Three strains from the same Slovenian salami, namely HNS21, HNS28 and HNS48 inhibited respectively 5, 6 and 7 log CFU/ml of *E. coli*. Proving their ability to inhibit the growth of $1x10^6$, $1x10^7$ and $1x10^8$ CFU/g of the targeted microorganism. On the other hand, only 8 strains of LAB were able to reduce 4 log CFU of *List. innocua* with only one strain (HNS48) able to reduce microbial cell load of 5 log CFU/ml, inhibiting $8x10^5$ CFU/ml *List. innocua*. Different experiments recently investigated the effect of different compounds derived from LAB as bioprotectants in food models. For example, Incili at al. (2023) tested the effect of paraprobiotics deriving from *Pediococcus acidilactici* to inhibit *E. coli* O157:H7, *Salmonella typhimurium* and *List. monocytogenes* in meatballs. They report that the concentration of paraprobiotics should be 10 times higher in food with respect to *in vitro* to achieve the same inhibitory effect, and that this phenomenon is probably due to the matrix effect. In our experiments we obtained a sensible reduction of pathogens by using live microorganisms, which suggests their efficacy in increasing safety of processed meat (Kürşad İncili et al., 2023). In Pisano et al. (2022), an inhibitory effect of LAB against *List. monocytogene*s is reported, where *Lactiplantibacillus plantarum* and *Lactococcus lactis* produced an inhibition zone of > 4 mm in cheese agar (Pisano et al., 2022).

By-products of LAB metabolism can allow the inhibition of undesired microorganisms, determined by means of a synergistic effect between different bioactive compounds and an adverse environment for the growth of harmful microbes. Among all bio-protectant acid compounds, lactic acid seems to be particularly effective (Barcenilla et al., 2022; Parlindungan et al., 2021). In fact, the highest reductions in log CFU values were achieved by the strains which caused the greater drop in pH values (Table 4).

From our results, it was also possible to notice that the strains with best inhibitory performances against E. coli*, caused the most relevant decrease of* List. innocua *concentrations. Nevertheless, lower values of* Listeria *reduction achieved in our experiments are supported also by literature. As an example, Wang et al. (2015), reported the necessity of an increased contact time between* List. monocytogenes *and lactic acid, with respect to* Salmonella *spp. and* E. coli*, to achieve the same level of inactivation (Wang et al., 2015).*

Table 10 Log reduction of E. coli and List. innocua and final pH in meat model used for the assays.

Strains	**Species**	**Target microorganisms Log_{10} CFU reduction**		**Final pH**	
		E. coli **ATCC 25922**	***List. innocua*** **UC8409**	***E. coli*** **ATCC 25922**	***List. innocua*** **UC8409**
1M8	*L. sakei*	1	1	4.16	4.15
1M24	*L. sakei*	4	4	3.71	3.71
1M51	*L. sakei*	4	3	3.63	3.63
2M7	*L. sakei*	3	2	3.94	3.96
2M9	*L. sakei*	4	4	3.72	3.68
IAL8	*L. sakei*	3	2	4.07	4.10
IAL18	*L. sakei*	1	0	4.32	4.08
IAL38	*L. sakei*	2	3	3.69	3.65
SN4	*L. sakei*	3	1	4.36	4.36
SN34	*L. sakei*	4	4	3.72	3.70
SN58	*L. sakei*	4	4	3.89	3.85
SN63	*L. sakei*	2	1	3.89	3.94
SN70	*L. sakei*	2	1	4.03	3.99
SWO10	*L. sakei*	4	4	3.65	3.70
SWO18	*L. sakei*	2	1	4.11	4.11
SWO29	*L. sakei*	3	2	3.75	3.74
SWO48	*L. sakei*	3	3	3.70	3.68
SWO60	*L. sakei*	2	2	3.91	3.90
SWO61	*L. sakei*	3	2	3.84	3.94
ESA21	*L. sakei*	4	3	3.85	3.85
ESA49	*L. sakei*	3	3	3.79	3.82
ESB2	*L. sakei*	4	4	3.83	3.83
ESB7	*L. sakei*	2	1	4.03	4.07
ESB14	*L. sakei*	4	4	3.69	3.70
ESB24	*L. sakei*	3	3	3.82	3.85
ESB53	*L. sakei*	1	1	3.98	4.19
ESB60	*L. sakei*	3	2	3.97	4.17
ESB67	*L. sakei*	4	2	3.95	4.06
ESE30	*L. sakei*	3	2	3.87	3.85
ESE41	*L. sakei*	2	1	3.97	4.03
ESE67	*L. sakei*	2	3	4.05	4.01
ESO8	*L. sakei*	2	3	4.04	4.00
ESO10	*L. sakei*	2	3	4.04	4.04
ESO23	*L. sakei*	2	3	4.01	3.98
ESO38	*L. sakei*	1	2	3.90	3.93
ESO47	*L. sakei*	3	2	3.93	3.92
ESO65	*L. sakei*	3	3	3.86	3.89
ECE2	*L. sakei*	3	1	3.87	3.91
ECO38	*L. sakei*	4	1	3.64	3.83
HNS21	*L. sakei*	6	4	3.86	3.84
HNS28	*L. sakei*	5	3	3.83	3.89
HNS48	*L. sakei*	7	5	3.77	3.78
HNS55	*L. curvatus*	2	3	4.12	4.14

HZK39	*L. sakei*	1	3	4.29	4.13
HZK42	*L. sakei*	4	3	3.91	4.04
HZK50	*L. sakei*	2	1	4.00	4.10

4.3. Presence of bacteriocin genes

To further characterize the isolated strains, the presence of genes coding for bacteriocins was investigated. Results showed that 25 strains out of 46 possessed almost one bacteriocin-producing gene, and specifically, 14 strains harboring one gene and 11 strains showing the presence of more than one (Table 6). Two strains deriving from a Slovenian smoked salami appeared to be particularly interesting for the presence of 4 different genes coding for bacteriocins, namely HZK39 and HZK42. In detail, HZK39 strain showed the presence of *spp*A, *spp*Q, *sak*T and *sak*X genes, while HZK42 possessed *sap*A, *spp*A, *sak*G and *sak*X, confirming the preliminary results obtained in the agar overlay assay and in the salami-food model. Conversely, among the 46 strains analyzed, 21 strains did not exhibit any genes encoding bacteriocins. Among the LAB strains investigated in this study, *sakX* (present in 20 out of 25 strains) and *sppA* (present in 12 out of 25 strains) were the most observed genes associated with bacteriocin production. The presence of genes to produce different bacteriocins is reported in the literature and is bound to the ability of strains to face competition from bacteria from other genera. Moreover, it is reported that genes coding for bacteriocins are often carried on plasmids, which can be acquired by microorganisms, supporting ability of different genera to produce the same bacteriocins (Lozo et al., 2021).

Table 11 Presence of genes detected by PCR and coding for bacteriocins in LAB strains object of the study.

Strains	**Species**	**Bacteriocin genes**						
		curA=*sap*A	*spp*A	*spp*Q	*sak*G	*sak*T	*sak*X	**Tot**
1M8	*L. sakei*							
1M24	*L. sakei*						X	1
1M51	*L. sakei*							
2M7	*L. sakei*				X		X	2
2M9	*L. sakei*							
IAL8	*L. sakei*							
IAL18	*L. sakei*							
IAL38	*L. sakei*							
SN4	*L. sakei*							
SN34	*L. sakei*						X	1
SN58	*L. sakei*		X				X	2
SN63	*L. sakei*							
SN70	*L. sakei*							
SWO10	*L. sakei*							
SWO18	*L. sakei*							
SWO29	*L. sakei*						X	1
SWO48	*L. sakei*							
SWO60	*L. sakei*							
SWO61	*L. sakei*						X	1

ESA21	*L. sakei*		X				X	2
ESA49	*L. sakei*							
ESB2	*L. sakei*		X				X	2
ESB7	*L. sakei*		X					1
ESB14	*L. sakei*							
ESB24	*L. sakei*						X	1
ESB53	*L. sakei*							
ESB60	*L. sakei*						X	1
ESB67	*L. sakei*						X	1
ESE30	*L. sakei*						X	1
ESE41	*L. sakei*							
ESE67	*L. sakei*						X	1
ESO8	*L. sakei*		X	X			X	3
ESO10	*L. sakei*							
ESO23	*L. sakei*							
ESO38	*L. sakei*							
ESO47	*L. sakei*						X	1
ESO65	*L. sakei*			X			X	2
ECE2	*L. sakei*							
ECO38	*L. sakei*	X	X				X	3
HNS21	*L. sakei*		X					1
HNS28	*L. sakei*		X					1
HNS48	*L. sakei*		X					1
HNS55	*L. curvatus*	X					X	2
HZK39	*L. sakei*		X	X		X	X	4
HZK42	*L. sakei*	X	X		X		X	4
HZK50	*L. sakei*		X	X				2

4.4. Growth and acidification performances of selected LAB strains under different NaCl conditions

Considering the isolation sources, the inhibitory activity against pathogens and the presence of genes coding for bacteriocins, 20 strains were chosen for further technological characterization (Table 1). In particular, the growth of 19 *L. sakei* and one *L. curvatus* strains at different temperatures and NaCl concentrations was determined by monitoring the changes in optical density (OD_{600}). For each condition and strain, the experimental data were modelled using the Gompertz equation. The estimated parameters are reported in Table S1, together with the maximum pH decrease. To highlight the variability among strains, Figure 1 reports Box and Whisker plots concerning the distribution of the parameter estimates in relation to NaCl concentration and the pH decrease. The presence of significant differences ($P \leq 0.05$) was tested with one-way ANOVA. Concerning the parameter A, a significant diminution of the median (the thick line inside the box) was observed in relation to salt (the predicted OD_{600} was 1.96 at 0%, 1.82 at 2.5% and 1.60 at 5%). Three strains were considered outliers when cultivated in the absence of salt, due to their low A final level (SN34, SWO10 and ESB2). The same strains were characterized by the lowest final A at NaCl 2.5%, while at the higher salt level (5%) the lower value for this parameter were observed in the strains SN34 and SN58. As expected, the values of μ_{max} decreased with the increase of salt concentration (median values 0.161, 0.105, 0.045 h^{-1} at 0, 2.5 and 5%, respectively). The strain SN34

presented the best performance at 5% of salt concentration and was considered as an outlier, but it was characterized by high μ_{max} also in the other conditions (Table S1). Concerning λ, the increase was particularly relevant when NaCl concentration was 5% (median at 24.35 h, compared with 13.48 h at 0% and 15.89 h at 2.5%). In absence of salt, the strains SWO61, ECE2 and HNS48 presented the shorter λ estimates. The data concerning the pH value after incubation, reflected the growth extent as determined through OD_{600}; in other words, the pH decrease with respect to the initial value (approx. 5.9) was inversely proportional to the A value estimated for growth. In particular, ANOVA did not reveal differences among the strains tested at 0 and 2.5% (median values of pH decreases -1.86 and -1.75 units, respectively), while the strains grown at 5% showed a significant lower value (median -1.63).

Concerning the effect of temperature on growth kinetics, the parameters of the Gompertz equation were estimated (Table S1) and their distributions are reported in Figure 2. The value of A was not significantly different at 20 and 30°C (median OD_{600} 1.96 and 1.93, respectively), while at 10°C it was lower (median 1.83). In addition, a *L. sakei* strain (CO38) did not grow at the lower temperature. Noteworthy, the estimates at 10°C were characterized by a greater variability, as demonstrated by the variability coefficient (CV) reported in Table S1. Three strains (SN34, SWO10 and SB2) presented the lower A values, independently on the temperature (the outliers at 20°C).

Temperature had a marked effect on median values of μ_{max} which significantly decreased passing from 30 to 10°C (0.268, 0.161 and 0.034 h^{-1}, respectively).

The length of λ showed an increase from 30 to 20°C, passing from 6.87 h to 13.48 h that, resulted not significant according to ANOVA. At 10°C the median value was 49.38 h with a strain with no growth and strain ESO23 characterized by an extremely long λ (147.28 h), considered as outlier. As already observed for the effect of salt, the final pH decrease obtained at the end of fermentation was related to the values of A. The final pH observed was not significantly different at 20 and 30°C (the median pH decrease was -1.86 and -1.92 respectively), while this value at 10°C was -1.50.

The data indicated positive performances of the strains in terms of growth at low temperature (with the exception of the strain ECO38 that was not able to grow at 10°C) and a good aptitude to multiply at 5% of salt, confirming the data reported for other *L. sakei* strains by Montanari et al. (2018), who tested the strain performances even at 5°C and 8% of salt (Montanari et al., 2018). In addition, Ammor et al. (2005) showed that 97% of *L. sakei* strains, isolated from traditional dry sausages, grew at 4°C, while 55% of them was able to grow in the presence of 6.5% of salt (Ammor et al., 2005). The increases of the variability under the most restrictive conditions, highlights the importance of these screening tests in order to select candidates for their use as potentially new starter cultures.

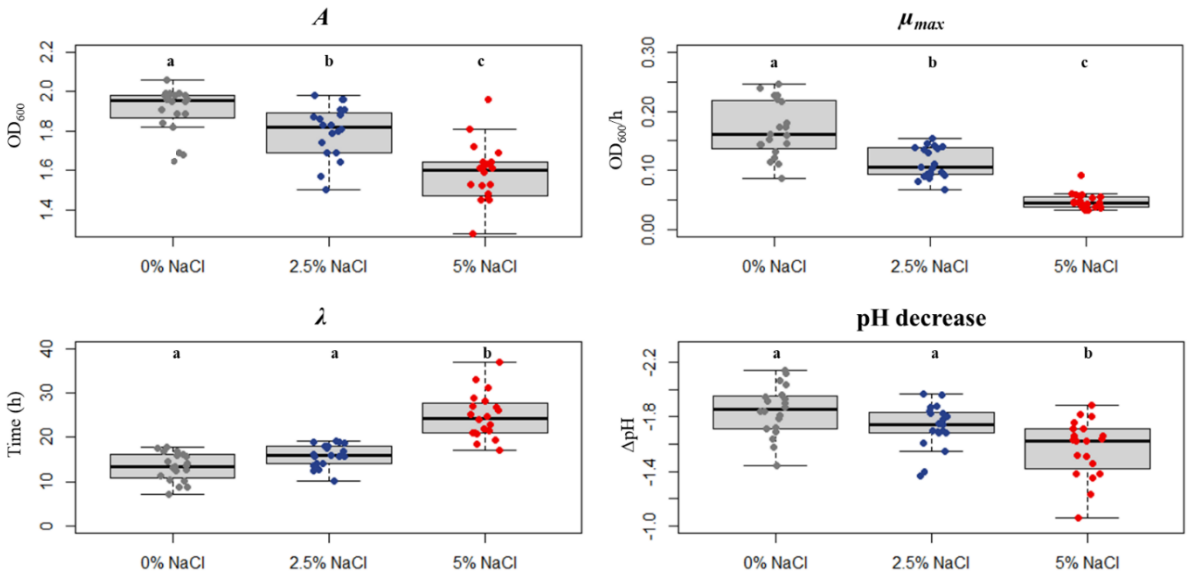

Figure 9 Box and Whisker plots representing the distribution of parameters estimated by Gompertz equation (A, μ_{max} and λ) of strain growth kinetics at different salt concentrations (0%, 2.5% and 5%). Final pH decrease under different conditions is also reported. In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ± 1.5 times the box height.

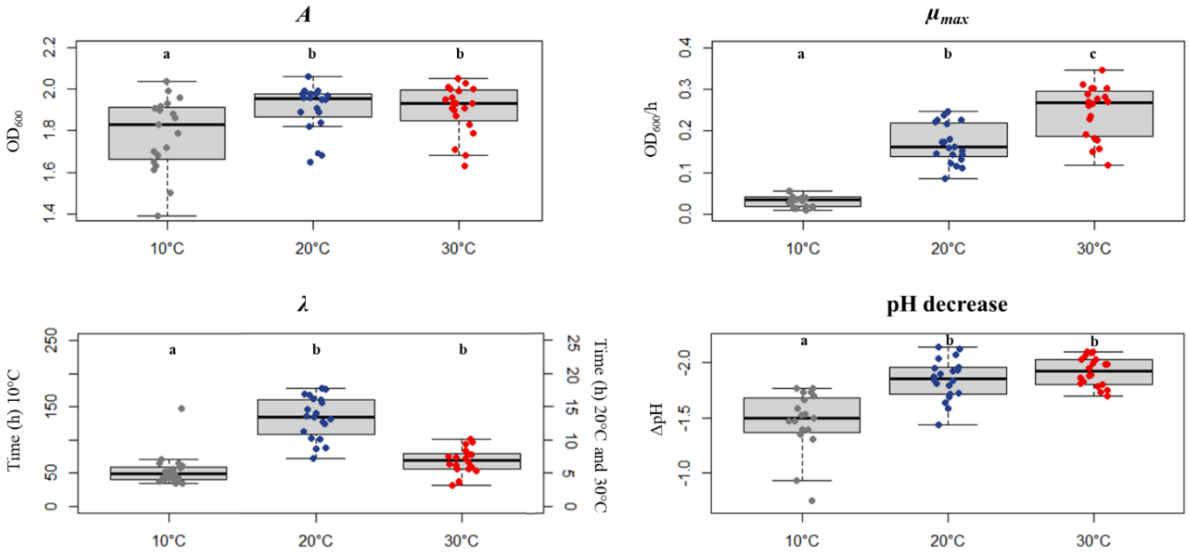

Figure 10 Box and Whisker plots that represent the distribution of parameters estimated by Gompertz equation (A, μ_{max} and λ) of strain growth kinetics at different incubation temperatures (10°C, 20°C and 30°C). Final pH decrease in the different conditions is also reported. In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ± 1.5 times the box height.

5. CONCLUSIONS

In this study, a total of 46 LAB strains were subjected to safety assessment to determine their non-amino-biogenic and non-antibiotic-resistant properties. Primarily, these strains belonged to the *L. sakei* species, with only one strain identified as *L. curvatus*. The purpose of this investigation was to identify potential autochthonous starter cultures and/or bio-protective food cultures. The first step to evaluate their antimicrobial activity against *E. coli* ATCC 25922 and *List. innocua* UC8409 revealed that all tested strains exhibited inhibitory effects on the growth of both target microorganisms, with strain-dependent differences. Generally, *E. coli* exhibited greater sensitivity to LAB inhibition, while *List. innocua* was comparatively less affected. Furthermore, the antimicrobial activity of LAB strains against the same target microorganisms was assessed using a fermented meat model, which confirmed the greater sensitivity of *E. coli* compared to *List. innocua*. However, the most effective strains demonstrated the ability to inhibit both target pathogens. Subsequently, the presence of bacteriocin encoding genes was investigated, with 24 *L. sakei* strains and the *L. curvatus* strain found to possess at least one bacteriocin encoding gene, predominantly sakacin X and sakacin P. These strains exhibited the highest antimicrobial activity against the target microorganisms *in vitro* and demonstrated an equivalent inhibitory potential in the fermented meat model. Considering the promising antimicrobial performances, the most favorable strains were selected for further technological analyses to assess their performances under varying salt concentrations and incubation temperatures. As anticipated, an observed reduction in the growth potential was noted as salt concentration increased or temperature decreased, resulting in lower growth rates and longer lag phases. The strain behavior exhibited significant variability, highlighting the phenotypic diversity previously reported for LAB species well-adapted to the meat environment. Based on these analyses, a number of LAB strains exhibiting favorable technological characteristics and significant antimicrobial potential were identified. These strains hold promise for potential application as starter or bio-protective cultures in meat-based food systems, including fresh or fermented sausages. Their utilization has the potential to enhance food quality, ensure microbiological safety, and impart distinctive attributes to the final product.

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7. AUTHORS CONTRIBUTIONS

Giovanni Milani: Methodology, Formal analysis. Giulia Tabanelli: Conceptualization, Writing - original draft, Supervision. Federica Barbieri: Data curation, Methodology, Formal analysis. Chiara Montanari: Data curation, Writing - original draft. Fausto Gardini: Conceptualization, Writing - review & editing. Mireya Viviana Belloso Daza: Formal analysis. Vincenzo Castellone: Writing - original draft. Marianna Bozzetti: Methodology. Pier Sandro Cocconcelli: Writing - review & editing. Daniela Bassi: Conceptualization, Supervision.

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10. SUPPLEMENTARY MATERIALS

Table S1: *Gompertz parameters (A, μ_{max} and λ) of Lat. sakei and Lat. curvatus strains growth at 20°C in presence of different salt concentrations (0, 2.5 and 5%) and at different incubation temperatures (10, 20 and 30°C) in absence of salt. Median, mean value, standard deviation (SD) and variability coefficient (CV) are also reported. The pH decrease at the end of incubation is also showed.*

	0% NaCl				2.5% NaCl				5% NaCl				10°C				20°C				30°C			
Strains	***A***	μ_{max}	**λ**	**pH**	***A***	μ_{max}	**λ**	**pH**	***A***	μ_{max}	**λ**	**pH**	***A***	μ_{max}	**λ**	**pH**	***A***	μ_{max}	**λ**	**pH**	***A***	μ_{max}	**λ**	**pH**
1M24	1.98	0.180	13.09	-1.84	1.81	0.155	16.02	-1.55	1.69	0.044	20.93	-1.38	1.83	0.039	52.12	-1.53	1.98	0.18	13.09	-1.84	1.91	0.278	5.30	-2.09
2M7	1.89	0.153	11.26	-1.90	1.83	0.094	14.14	-1.69	1.53	0.045	26.13	-1.52	1.86	0.031	56.33	-1.36	1.89	0.153	11.26	-1.90	1.93	0.178	7.41	-1.83
2M9	1.89	0.221	16.18	-1.58	1.74	0.141	18.73	-1.40	1.45	0.059	33.13	-1.23	1.92	0.054	33.81	-0.93	1.89	0.221	16.18	-1.58	1.90	0.282	7.45	-1.78
IAL8	1.84	0.161	14.63	-1.64	1.83	0.130	16.06	-1.61	1.62	0.055	22.91	-1.64	1.70	0.034	70.42	-1.39	1.84	0.161	14.63	-1.64	1.83	0.235	7.17	-1.75
SN34	1.68	0.247	16.81	-2.04	1.50	0.137	18.84	-1.68	1.28	0.091	37.10	-1.06	1.79	0.042	60.10	-1.31	1.68	0.247	16.81	-2.04	1.63	0.303	7.78	-2.03
SN58	1.91	0.174	12.38	-1.72	1.69	0.090	14.07	-1.74	1.16	0.038	21.53	-1.51	1.90	0.037	40.32	-1.70	1.91	0.174	12.38	-1.72	2.03	0.271	3.58	-1.86
SWO10	1.65	0.239	16.03	-1.44	1.57	0.139	17.95	-1.37	1.52	0.054	26.71	-1.38	1.58	0.070	34.00	-0.75	1.65	0.239	16.03	-1.44	1.68	0.302	7.98	-1.80
SWO61	1.98	0.114	7.14	-2.07	1.96	0.094	10.06	-1.75	1.72	0.058	20.76	-1.46	1.72	0.025	46.55	-1.35	1.98	0.114	7.14	-2.07	1.87	0.313	6.21	-1.95
ESB2	1.69	0.146	13.40	-1.71	1.64	0.104	19.09	-1.78	1.63	0.033	29.01	-1.80	1.50	0.013	64.20	-1.50	1.69	0.146	13.40	-1.71	1.71	0.262	9.40	-1.70
ESB14	1.98	0.110	8.73	-1.93	1.87	0.091	14.02	-1.68	1.61	0.045	24.67	-1.35	1.99	0.016	41.01	-1.52	1.98	0.11	8.73	-1.93	1.91	0.157	5.96	-2.10
ESE30	1.95	0.217	15.58	-1.95	1.69	0.135	18.94	-1.96	1.48	0.047	31.31	-1.76	2.04	0.041	49.38	-1.47	1.95	0.217	15.58	-1.95	1.99	0.265	7.77	-1.98
ESO8	1.97	0.227	13.99	-1.69	1.80	0.142	15.76	-1.70	1.46	0.043	28.32	-1.62	1.63	0.018	51.10	-1.73	1.97	0.227	13.99	-1.69	2.00	0.289	6.28	-1.73

ESO23	1.82	0.174	16.94	-1.92	1.69	0.096	18.05	-1.83	1.45	0.043	27.12	-1.71	1.68	0.017	147.28	-1.39	1.82	0.174	16.94	-1.92	1.79	0.271	8.42	-1.88
ESO65	1.96	0.160	10.04	-2.12	1.91	0.111	12.77	-1.80	1.64	0.060	21.13	-1.62	1.91	0.027	40.46	-1.77	1.96	0.16	10.04	-2.12	2.01	0.302	3.66	-2.03
ECE2	1.96	0.122	8.68	-1.87	1.88	0.106	13.61	-1.70	1.59	0.048	19.30	-1.66	1.88	0.008	56.81	-1.47	1.96	0.122	8.68	-1.87	1.93	0.229	5.66	-2.06
ECO38	1.99	0.086	17.82	-1.96	1.96	0.068	16.88	-1.97	1.81	0.036	18.49	-1.89	-*	-	-	-	1.99	0.086	17.82	-1.96	2.05	0.182	9.62	-2.10
HNS48	1.99	0.143	10.30	-1.79	1.86	0.097	12.53	-1.84	1.61	0.037	17.11	-1.66	1.93	0.055	64.82	-1.73	1.99	0.143	10.30	-1.79	1.95	0.191	5.62	-1.98
HZK39	1.99	0.144	17.64	-1.81	1.91	0.082	17.54	-1.88	1.96	0.037	24.03	-1.82	1.61	0.013	42.44	-1.66	1.99	0.144	17.64	-1.81	2.00	0.149	10.16	-1.89
HZK42	1.95	0.132	12.64	-1.84	1.79	0.087	15.69	-1.87	1.53	0.033	21.90	-1.71	1.65	0.039	34.72	-1.58	1.95	0.132	12.64	-1.84	1.93	0.117	6.57	-1.81
HNS55	2.06	0.227	13.56	-2.14	1.98	0.146	15.72	-1.83	1.64	0.056	25.13	-1.63	1.96	0.037	43.63	-1.77	2.06	0.227	13.56	-2.14	1.96	0.347	3.18	-1.99
Median	1.96	0.161	13.48	-1.86	1.82	0.105	15.89	-1.75	1.60	0.045	24.35	-1.63	1.83	0.034	49.38	-1.50	1.96	0.161	13.48	-1.86	1.93	0.268	6.87	-1.92
Mean value	1.91	0.169	13.34	-1.85	1.80	0.112	15.82	-1.73	1.57	0.048	24.84	-1.57	1.79	0.032	54.18	-1.47	1.91	0.169	13.34	-1.85	1.90	0.246	6.76	-1.92
SD	0.11	0.047	3.19	0.18	0.13	0.026	2.50	0.16	0.17	0.013	5.14	0.21	0.16	0.016	25.04	0.27	0.11	0.047	3.19	0.18	0.12	0.063	1.95	0.13
CV	6.02	27.87	23.91	9.86	7.37	22.92	15.82	9.24	11.07	27.74	20.68	13.37	8.71	50.56	46.22	18.28	6.02	27.87	23.91	9.86	6.20	25.76	28.89	6.81

11. *: no growth was observ

CHAPTER 8

Companilactobacillus alimentarius: an extensive characterization of strains isolated from spontaneous fermented sausages

Companilactobacillus alimentarius: an extensive characterization of strains isolated from spontaneous fermented sausages.

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1. ABSTRACT

Companilactobacillus alimentarius is a facultatively heterofermentative lactic acid bacterium (LAB) that is a significant constituent within the microbiota of various traditional fermented foods exerting several functions in fermentative or ripening processes. This species has been isolated from Spanish fermented sausages, where its frequency of isolation was comparable to those of *Latilactobacillus sakei* and *Latilactobacillus curvatus*. Despite to its presence in several niches, ecological information on this species is still scarce and only few publications report information about its safety features (i.e. antibiotic resistance). Since studies on *C. alimentarius* concern the analysis of a few individual traits regarding this species, a more extensive work on a larger number of isolates from the same matrix have been performed to allow a clearer interpretation of their phenotypic and technological characteristics. Specifically, 14 strains of *C. alimentarius* isolated from Mediterranean spontaneously fermented sausages, have been screened for their safety and technological characteristics (such as antibiotic resistance, biogenic amine production, inhibiting potential, growth at different temperatures and NaCl concentrations) and with phenotype microarrays with the aim to elucidate their potential role and contribution to sausage fermentation and ripening.

In general, a wide variability was observed in relation to the parameters considered. Several of the tested strains were able to produce histamine, tyramine and putrescine while the antibiotic resistance greatly varied according to the strains, with the exception of vancomycin. In addition, *C. alimentarius* strains showed a relevant potential to grow in conditions of salt and temperature mimicking those found in fermented foods. In particular, the growth at 10 °C and in the presence of salt can explain the presence of *C. alimentarius* in sausages and its adaptation to fermented meat environment in which low temperature can be applied during ripening. The differentiation of the phenotypic profile reflected the environmental conditions that influenced the isolation source, including those derived by the raw materials.

Given the species frequent association with spontaneous fermentations or the ripening microbiota of various products, despite not being intentionally used as starter cultures, the data presented in this study contribute to a deeper comprehension of their role, both advantageous and detrimental, in numerous significant fermented foods.

2. INTRODUCTION

Companilactobacillus (formerly *Lactobacillus*) *alimentarius* is a Gram are significant constituents within the microbiota of various traditional positive facultatively heterofermentative lactic acid bacterium (LAB) for fermented foods and fulfil crucial functions in fermentative or ripening processes (Bassi et al., 2022; Fujimoto et al., 2019; García Fontan et al., ´ 2007a, 2007b). Their optimal temperature ranges between 25 and 30 °C and they can grow at 15 °C and at 37 °C using pentoses, hexoses and disaccharides as carbon sources; the genome size is 2.34 Mbp and the mol% G + C content of DNA is 35.4. (Zheng et al., 2020). In addition, the species *C. alimentarius* is recognized under the Qualified Presumption of Safety (QPS)

status by the European Food Safety Authority (EFSA) (EFSA, 2020) and is part of the inventory of microbial food cultures with safety demonstration in fermented food products (Bourdichon et al., 2022).

Despite the relative scarcity of information on its physiological role, the presence of *C. alimentarius* is particularly relevant in fermented foods obtained from vegetable matrices (Guo et al., 2022; Liang et al., 2022). This species has been found in wheat (Randazzo et al., 2005), rye (Corsetti et al., 2001) and whole soft wheat sourdoughs (Taccari et al., 2016), although not as a dominant population. According to Fujimoto et al. (2019), *C. alimentarius* was the second species, after *Levilactobacillus brevis*, isolated from Japanese sourdough. It is also present in some traditional fermented products, such as Tarhana, obtained from wheat flour, yogurt, vegetables, and spices (Ozel et al., 2020¨). Other spontaneously fermented vegetables are characterized by the presence of this species. For example, during the fermentation of pickled chayote (*Sechium edule*), *C. alimentarius* was the prevalent species, accompanied by other LAB and yeasts (Shang et al., 2022), as well as in other Chinese fermented foods obtained from *Brassica juncea*, *Raphanus sativus* and *Capsicum annuum* (Liu and Tong, 2017).

In addition to vegetable matrices, this species has been often isolated from fermented meats. In their survey on the microbial communities of European fermented sausages, Van Reckem et al. (2019) found only sporadic presence of *C. alimentarius*. Nevertheless, strains belonging to this species were isolated from Spanish fermented sausages, such as Androlla (García Fontan et al., 2007a´) and Botillo (García Fontan et al., ´ 2007b), where their frequency of isolation was comparable to those of *Latilactobacillus sakei* and *Latilactobacillus curvatus*. The relevant presence of *C. alimentarius* was observed also in Turkish fermented sausages (sucuk) by Gürakan et al. (1995) and Kesmen et al. (2012). A potential probiotic strain of *C. alimentarius* has also been used as starter cultures for the production of fermented Scandinavian sausages by Klingberg et al. (2005).

C. alimentarius has also been associated with dairy products. An interesting work of Cardinali et al. (2017) demonstrated that this bacterium was present in the phyllosphere of *Carlina acanthifolia*, a plant traditionally used for vegetable rennet production, and is able to pass through the rennet, in the goat milk, affecting the early bacterial dynamics during cheesemaking. This species has been also tested as probiotic for promoting goat intestinal health and producing milk with higher concentrations of unsaturated fatty acid (Apas et al., 2015´).

From a bioprotective perspective, certain strains of *C. alimentarius* have exhibited the capability to produce bacteriocins, which have been subjected to preliminary investigations to assess their efficacy against *Bacillus* spp., the causative agents of bread rope (Mentes¸ et al., 2007). Hu et al. (2017) demonstrated the production of a bacteriocin (lactocin MM4) with a broad inhibitory range towards several Gram-positive and Gram-negative bacteria as well as against some fungi. In addition, it was demonstrated that members of this species can produce phenyllactic and 4-hydroxy-phenyllactic acids, metabolites active against spoiling moulds (Valerio et al., 2004). The bioprotective effect of the strain Flora- carn L2 against *Listeria monocytogenes* has been described in meat (Juven et al., 1998). Another study

demonstrated the capacity of this species together with *Staphylococcus xylosus* to act as protective cultures in under vacuum sliced cooked ham (Kotzekidou and Bloukas, 1998). On the other hand, in fishery products *C. alimentarius* wasresponsible for herring spoilage, causing bulging of lids and gas formation (Lyhs et al., 2001).

Concerning safety aspects, few publications report information on the antibiotic resistance profile of this species. According to Gevers et al. (2003), two strains of this species harbour a plasmid-located *tet(M)* gene with transfer capacity to *Enterococcus faecalis*, while in a study of Campedelli et al. (2019) members of this species were found to display *tet*(S) gene and *Isa* gene encoding for resistance to clindamycin. Despite its diffusion in several spontaneous fermented foods, few information is available concerning its ability to produce biogenic amines (BAs). Previous studies reported that one strain isolated from fermented food, was able to produce tyramine (Straub et al., 1995), while another study showed no decarboxylase activity in a strain isolated from Himalayan fermented foods (Dewan and Tamang, 2007), as well as in a strain from table olives (Yalçınkaya and Bas¸ yigit Kılıç, 2019˘). Recently, a metagenomics study on the microbial communities present in Mediterranean spontaneously fermented sausages showed that *Companilactobacillus* spp. was among the prevalent genera in salamis, some of them characterized by the presence of relevant amounts of BAs (Barbieri et al., 2021).

To date, studies on *C. alimentarius* concern the analysis of a few individual traits regarding this species, but there is a lack of more extensive works on a larger number of isolates from the same matrix allowing a clearer interpretation of their phenotypic and technological characteristics. The aim of this work was to perform a wider characterization of 14 strains of *C. alimentarius* isolated from Mediterranean fermented sausages. In order to elucidate the potential role and contribution of *C. alimentarius* strains in sausage fermentation and ripening, all strains were firstly screened for phenotype microarrays and then characterized for aspects concerning safety and technological issues (such as antibiotic resistance, BAs production, inhibiting potential, growth at different temperatures and NaCl concentrations).

3. MATERIALS AND METHODS

3.1. *Companilactobacillus alimentarius* microbial strains

The strains used in this study were isolated from Spanish spontaneously fermented sausages (Andalusia region), in which the presence of *Companilactobacillus* spp. was detected through metagenomic analysis (Barbieri et al., 2021) and strains belonging to the species *C. alimentarius* were isolated from the ripened products (Bassi et al., 2022). The list of the 14 strains considered in this study is reported in Table 1.

Pure cultures were stored at − 20 °C in De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) containing 20 % glycerol (Sigma Aldrich) until further analyses.

Source of isolation	Relative abundance (%) of *Companilactobacillus* detected by metagenomic analysis	% of isolation of *C. alimentarius* on all the isolated strains	*C. alimentarius* strains studied
Chorizo Berchules ´	45.0	87.5	CB1, CB6, CB8, CB16, CB22, CB31, CB36, CB41, CB43
Chorizo Ecija ´	34.5	4.5	CE49
Chorizo Olvera	5.3	57.0	CO12, CO24, CO50
Salchichon ´ Ecija ´	55.3	12.5	SE14

Table 1*. Source of isolation of the 14 strains considered in this study, relative abundance (%) of Companilactobacillus spp. detected by metagenomic analysis and percentage of isolation of C. alimentarius on all the isolated strains (adapted from Barbieri et al. (2021) and Bassi et al. (2022).*

3.2. Antimicrobial activity against food-borne pathogens

The antimicrobial activity of the 14 *C. alimentarius* strains was evaluated through an agar spot test against *L. monocytogenes* Scott A and *Salmonella enterica* serovar Enteritidis 155, belonging to the collection of the Department of Agricultural and Food Science of the University of Bologna.

The foodborne pathogens were grown into Brain Heart Infusion (BHI) medium (Oxoid), while LAB strains into MRS agar (Oxoid). Each culture was incubated overnight at 30 °C. Target pathogens were inoculated in BHI soft agar (0.7 %) plates to obtain a final concentration of 6 log CFU/ml to form a bacterial lawn. Once the plate was dried, 10 µl drop of each *C. alimentarius* strain cultures were spotted onto plates. The samples were observed after 24 h of incubation at 30 °C and the absence/presence of inhibition zones was evaluated. The inhibitory activity was expressed based on the diameter halo around the spot: + (≤0.5 cm), ++ (0.5–2 cm), +++ (>2 cm), or - (no halo). Cell free supernatants (CFS) of *C. alimentarius* strains that showed an antimicrobial activity were collected after centrifugation at 6000 rpm for 10 min and filtration with polyethersulfone (PES) membrane (Merck Millipore, Carrigtwohill, Ireland) with a pore size of 0.22 µm. The CFS were collected in sterile microcentrifuge tubes and tested, both unmodified and neutralised at pH 6.5 with NaOH 1 M, through the well technique against the same pathogens. Once the indicator strain has grown, the appearance of inhibition halos around the wells was observed and measured in millimeters to detect the inhibitory activity. Three independent tests were carried out, and each sample was tested in duplicate.

3.3. Phenotype microarray of *C. alimentarius* strains

The screening of *C. alimentarius* strains was performed with a phenotype microarray (OmniLog®, Biolog, Inc., Hayward, USA), using Gen III MicroPlate, according to the manufacturer's instructions. For each

strain, colonies cultured onto MRS agar medium were resuspended into inoculating fluid C (Biolog, Inc.), until reaching a microbial cell concentration from 90 % to 98 % T (the light transmittance measured by OmniLog® turbidimeter). Briefly, 100 µl of each cell suspension were inoculated into the MicroPlate wells and incubated at 30 °C for 96 h in accordance with growth characteristics. Measurement of strain metabolism was assessed by colorimetric redox assay and all MicroPlates were read every 15 min. The data were collected with the OmniLog® and companion computer software.

3.4. Antibiotic-resistance profile

Antibiotic-resistance profile of the strains was assessed considering EFSA indications (EFSA, 2012). A minimum inhibitory concentration (MIC) test to evaluate the resistance to ampicillin (Amp), chloramphenicol (Chl), clindamycin (Cli), erythromycin (Ery), gentamicin (Gen), tetracycline (Tet), kanamycin (Kan) and streptomycin (Str) was performed using micro dilution technique in the recommended Lymphocyte Separation Medium (LSM) medium (Iso-Sensitest™ broth 90 % and MRS broth 10 %; ThermoFisher Scientific) (ISO, 2010). Results were collected after 48 h of incubation at 30 °C and the presence of resistance for each antibiotic is defined according to the cut off reported by EFSA: Gen = 16, Kan = 64, Str = 64, Tet = 8, Ery = 1, Clin = 1, Chlor = 4, Amp = 4 (EFSA, 2012).

3.5. Biogenic amines production

The amino biogenic potential of *C. alimentarius* strains was tested through the screening in Bover-Cid-Holzapfel medium (BC) (Bover-Cid and Holzapfel, 1999). All strains were pre-cultivated in MRS broth and then inoculated in BC broth, supplemented with the biogenic amine (BA) precursors (histidine, tyrosine, ornithine or lysine) and incubated at 30 °C for 72 h. The supernatants of presumptive positive strains were collected and stored at − 20 °C, until the HPLC analysis. After a dansyl- chloride derivatization (Sigma-Aldrich, St Louis, USA), samples were injected into HPLC Agilent Technologies 1260 Infinity with the automatic injector (G1329B ALS 1260, loop of 20 µl), equipped with a C18 Waters Spherisorb ODS-2 (150 × 4.6 mm, 3 µm) column and a UV detector (G1314F VWD 1260) set at 254 nm, to confirm the BA production (histidine, tyramine, putrescine and cadaverine) according to the method reported by Montanari et al. (2023). The BA amount was measured with reference to a calibration curve obtained through the injection of dansyl-chloride-derivatized BA standards. Under the adopted conditions, the detection limit for all compounds was 3 mg/l. All the analyses were performed in triplicate.

3.6. Growth performances in presence of different salt concentrations and at different incubation temperatures

The growth performances of *C. alimentarius* strains were evaluated in relation to different salt concentrations (0 %, 2.5 % and 5 % NaCl) at 20 °C and at different incubation temperatures (10 °C, 20

°C and 30 °C). They were pre-cultivated in MRS broth for 24 h at 30 °C and then inoculated at a final concentration of 5 log CFU/ml in the different media chosen for the analyses. Their growth was monitored through the variation of optical density at 600 nm (OD_{600}) with time (t), measured with an UV-VIS spectrophotometer 6705 UV–Vis (Jenway, Stone, UK). The collected data were elaborated with Gompertz Eq. (1), as modified by Zwietering et al. (1990):

$$OD_{600} = A \cdot e^{-e^{((\frac{\mu max \cdot e}{A}) \cdot (\lambda - t) + 1)}} \quad (1)$$

where A represent the maximum OD600 value reached, μmax is the maximum OD600 increase rate (OD600 h-1) and λ is the lag phase (h).

Moreover, pH values were also monitored overtime by pH-meter Basic 20 (Crison Instruments). The data were modelled with the same equation modified as follow (Eq. (2)):

$$pH = k + A_{pH} \cdot e^{-e^{((\frac{\mu pHmax \cdot e}{A_{pH}}) \cdot (\lambda_{pH} - t) + 1)}} \quad (2)$$

where k is the higher asymptote of the curve (initial pH), ApH is the lower asymptote of the curve (final pH decrease), $\mu pHmax$ is the maximum pH decrease rate (pH h-1) and λpH is the lag phase (h).

3.7. Statistical analysis

The parameters of the OD_{600} and pH curves were estimated using Statistica 8.0 software (StatSoft Inc., Tulsa, USA). The distribution of the modelled parameters was tested with ANOVA to define statistically significant differences. Statistical differences were considered significant at a level of $p \leq 0.05$ using the Tukey test. ANOVA and Box and Whiskers plots were obtained by using the statistical software R (R Core Team, 2020).

4. RESULTS AND DISCUSSION

4.1. Antimicrobial activity of the *C. alimentarius* strains against *Salmonella* and *Listeria*

The 14 strains of *C. alimentarius* were tested for their capability to counteract the growth of *L. monocytogenes* ScottA and *S.* Enteritidis 155.

The cell suspensions of all 14 strains showed a similar inhibition against the target pathogens, being in general slightly more active against *S*. Eenteritidis 155 rather than *L. monocytogenes* (0.5–2 cm halo diameter versus ≤0.5 cm). The same observations can be done for CFS (data not shown), but this activity was not present after pH neutralization, indicating that the bioactivity was only due to acidification and no specific bacteriocins against the target pathogens were produced.

4.2. Screening using phenotype microarray

In Table 2, the results of the GenIII MicroPlate test concerning carbon sources and chemical sensitivity are reported. Fifty-nine tests resulted negative for all the strains (data not shown). Concerning the carbon sources, 12 of them were used by at least one strain. In particular, all strains grew on α-D-glucose and the majority was able to use D-mannose (with the exception of CB36, CB41 and CE49), D-fructose (except CB36 and CE49) and *N*-acetyl-D-glucosamine (except CB31 and CE49). D- maltose and α-D-lactose were fermented only by CB6, which metabolised also D-trehalose (with SE14) and showed a weak growth on D-galactose. The strain SE14 was the only able to grow on D-cellobiose, while sucrose was fermented by CB22, CB43, CO12 and, at a lesser extent, by SE14 and CB16. The strains CB43, CB22, CO24, CO50 and SE14 used D-salicin and β-methyl-D-glucoside as carbon sources. According to Randazzo et al. (2005), the four strains of *C. alimentarius* isolated from sourdoughs were all able to ferment glucose, fructose, maltose and sucrose, while according to Hu et al. (2017) the strain FM-MM$_4$ fermented glucose, fructose, galactose, trehalose and lactose. García Fontan et al. (2007b) ´ found that almost all the strains (96 %) of this species isolated from a Spanish fermented sausage (*Botillo*) can ferment sucrose, but none of them fermented lactose.

The growth in the presence of chemical sensitivity showed that all the strains were able to grow at pH 6 and 5 and when NaCl was added at 1 and 4 %, as well as with sodium lactate at 1 %. Only the strain CO50 grew also at 8 % NaCl. Concerning antibiotics, all the strains grew in the presence of vancomycin, nalidixic acid and fusidic acid, while four of the strains could grow with minocycline (CB8, CB16, CB31 and CB36). All the strains gave a positive response in the presence of tetrazolium violet, tetrazolium blue, potassium tellurite and sodium butyrate, but none with lithium chloride.

Strains	Carbon sources												Limiting conditions																				
	D-Maltose	D-Trehalose	D-Cellobiose	Sucrose	α-D-Lactose	β-Methyl-D-Glucoside	D-Salicin	N-Acetyl-D-Glucosamine	α-D-Glucose	D-Mannose	D-Fructose	D-Galactose	pH 6	pH 5	1% NaCl	4% NaCl	8% NaCl	1% Sodium Lactate	Fusidic Acid	D-Serine	Troleandomycin	Rifamycin SV	Minocycline	Lincomycin	Guanidine HCl	Niaproof 4	Vancomycin	Tetrazolium Violet	Tetrazolium Blue	Nalidixic Acid	Lithium Chloride	Potassium Tellurite	Aztreonam	Sodium Butyrate	Sodium Bromate
CB1	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-
CB6	+	+	-	-	+	-	-	+	+	+	+	±	+	+	+	+	-	+	±	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+
CB8	-	-	-	-	-	±	-	+	+	+	+	-	+	+	+	±	-	+	+	±	-	-	+	-	-	-	+	+	+	+	-	+	+	+	-
CB16	-	-	-	±	-	-	-	+	+	+	+	-	+	+	+	±	-	+	+	-	-	-	+	-	-	-	+	+	+	+	-	+	+	+	-
CB22	-	-	-	+	-	±	+	+	+	+	±	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+
CB31	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	±	-	+	+	+	-	-	+	-	-	+	+	+	+	+	-	+	+	+	-
CB36	-	-	-	-	-	-	-	+	+	-	-	-	+	+	+	±	-	+	+	-	-	-	+	-	-	-	+	+	+	±	-	+	-	+	±
CB41	-	-	-	-	-	±	-	+	+	-	+	-	+	+	+	±	-	+	+	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	-
CB43	-	-	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	±	-	-	-	-	+	+	+	+	-	+	+	+	-
CE49	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-
CO12	-	-	-	+	-	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-
CO24	-	-	-	-	-	+	±	±	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	-
CO50	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+
SE14	-	±	±	±	-	±	±	±	±	±	±	-	±	±	±	±	-	±	±	-	-	-	-	-	-	-	±	±	±	±	-	±	±	±	-

4.3. Antibiotic resistance

The 14 strains of *C. alimentarius* were tested for their antibiotic resistance by considering the 8 antibiotics indicated by EFSA (2012) for facultative heterofermentative lactobacilli. The results are reported in Table 3. The strain CB8 was the only one resistant to Kan, Tet and Ery. The same strain was also resistant to Chl, together with CB43 and CO12. Two strains were resistant to Gen (CO12 and SE14, 14.2 % of the total), three to clindamycin (CB41, CO12 and CO24, 21.3 %) and four to Str (CE49, CO12, CO50 and SE14, 28.4 %), while all the strains were sensitive to Amp. Overall, 6 strains were sensitive to all antibiotics considered and five were resistant to one antibiotic. The strains SE14 was resistant to two antibiotics while the strains CB8 and CO12 were resistant to four of the eight antibiotics tested.

In general, also for LAB species recognized as QPS (including C. alimentarius) and involved in food fermentations, antibiotic resistance should be assessed (Campedelli et al., 2019; Colautti et al., 2022; Klingberg et al., 2005). However, few studies are available concerning this aspect in C. alimentarius (Gevers et al., 2003).

Fermented meats can be a reservoir of antibiotic resistant LAB strains and the genes responsible for resistance can be horizontally transmissible to other species, including pathogens (Belloso Daza et al., 2022). Due to these factors, recent reports from EFSA suggest the need for further investigations on LAB involved in food fermentations (EFSA, 2012, 2021).

The resistance against aminoglycosides like gentamicin, streptomycin and kanamycin has a chromosomal origin in many LAB, even if its transmissibility through mobile genetic elements has been described in lactobacilli and enterococci (Werner, 2012; Zarzecka et al., 2022; Rozman et al., 2023). However, according to the data obtained in the present work, the resistance to this class of antibiotics did not seem to be a species characteristic and could be linked to genetic mobile elements. According to Campedelli et al. (2019), the resistance of type-strains assigned to the genus Companilactibacillus (formerly Lactobacillus alimentarius group) was approx. 20 % for gentamicin, 45 % for streptomycin and 80 % for kanamycin, while in this case, a percentage of 14.3 %, 28.6 % and 7.1 % was observed, respectively, for the same antibiotics. Concerning tetracycline, Gevers et al. (2003) found a plasmid located tetM gene in a strain of C. alimentarius isolated from fermented sausages and demonstrated the possibility to transfer this plasmid to a strain of Ent. faecalis. The same gene was found in several strains belonging to the species Lat. sakei, Lat. curvatus, Lactiplantibacillus paraplantarum, Lcb. paracasei from meat fermentations (Bassi et al., 2022; Fraqueza, 2015; Zonenschain et al., 2009). Also in this case, the incidence of resistant strains (7.1 %) was lower than that observed by Campedelli et al. (2019) for species of the genus Companilactibacillus, which were characterized by approx. 40 % of resistance. Also, for the macrolide erythromycin, plasmid associate genes (erm, msr and mef genes) responsible for acquired resistance has been found in lactobacilli (Leclercq and Courvalin, 1991; Zonenschain et al., 2009; Comunian et al., 2010) and the possibility to

transfer this plasmid to other LAB (Ent. faecalis) has been demonstrated (Nawaz et al., 2011). However, no information concerning specifically Companilactobacillus is available in literature. In this study, the percentage of clindamycin resistant strains (21.4 %) confirmed the results reported for Italian sausages by Federici et al. (2014), while the resistance to chloramphenicol (21.4 %) was higher than those reported in other similar studies (Aymerich et al., 2006).

Since this investigation cannot clarify the transmissibility or the type of mechanisms responsible for the resistance observed, further studies are needed to understand if these bacteria, present as component of the ripening microbiome of sausages and many other fermented products, can be responsible for the diffusion of antimicrobial resistance (AMR) genes to other microbial species.

Table 3. *Antibiotic-resistance profile of Companilactobacillus alimentarius strains, assessed following EFSA*

Strains	Gen	Kan	Str	Tet	Ery	Clin	Chlor	Amp
CB1	16	64	64	8	0.125	1	2	4
CB6	2	2	16	1	0.125	0.032	2	0.5
CB8	4	**128**	16	**16**	**4**	0.125	**8**	2
CB16	8	16	32	4	0.5	0.125	4	1
CB22	4	16	32	2	0.5	0.5	4	1
CB31	8	4	16	4	0.25	0.063	4	1
CB36	8	16	16	2	0.25	0.032	4	1
CB41	4	16	16	2	0.5	**4**	2	1
CB43	2	16	64	4	1	1	**8**	2
CE49	16	64	**128**	1	0.25	1	4	2
CO12	**32**	64	**128**	2	1	**4**	**8**	4
CO24	4	16	32	2	0.25	**4**	2	2
CO50	4	16	**256**	2	0.25	0.125	2	1
SE14	**32**	64	**256**	2	1	1	4	2

indications (Gen = Gentamicin; Kan = Kanamycin; Str = Streptomycin; Tet = Tetracycline; Ery = Erythromycin; Clin = Clindamycin; Chlor = Chloramphenicol; Amp = Ampicillin). The MIC value are reported and the presence of resistance for each antibiotic is highlighted in bold (cut off: Gen = 16, Kan = 64, Str = 64, Tet = 8, Ery = 1, Clin = 1, Chlor = 4, Amp = 4).

4.4. Biogenic amine production

The strains of C. alimentarius were isolated from sausages in which tyramine (from 67 to 202 mg/kg), putrescine (from 79 to 156 mg/kg) and, as far as one sample, histamine (174 mg/kg) were detected (Barbieri et al., 2021; Bassi et al., 2022). For this reason, the strains were tested for their ability to produce

BA in BC medium. The amounts of each BA detected after 72 h of incubation in BC medium is reported in Table 4.

Nine strains out of 14 (64.3 %) produced tyramine, even if at different extent. Four strains (CB1, CB6, CO12 and CO24) produced low amounts of this aromatic BA, ranging from 61.5 to 15.1 mg/l, while the other strains accumulated high level of tyramine. In fact, the strains CB8, CB16 and CB41 produced concentration of tyramine ranging from 386.1 to 489.2 mg/l, while extremely high concentrations were produced by the strains CE49 (745.0 mg/l) and CB36 (1156.6 mg/l).

Histidine decarboxylase activity was found in 4 strains. Two of them were high producers of histamine (>600 mg/l): CB16 and SE14. Minor amounts were detected for the strains CB1 and CE49, both characterized also by the presence of tyrosine decarboxylase. Putrescine was accumulated only by CB6 and CB16. Thus, this last strain was positive for the formation of three BA. Cadaverine was never detected. Finally, 4 strains did not show any decarboxylase activity (CB22, CB31, CB43 and CO50).

Species belonging to LAB are known to be the most important tyramine producer in fermented foods (Barbieri et al., 2019; Latorre-Moratalla et al., 2017). Tyrosine decarboxylase is extremely diffused and active among enterococci (Bargossi et al., 2015; Gatto et al., 2016; Ladero et al., 2012).

Nevertheless, the ability to produce tyramine is a strain characteristic of many species belonging to facultatively heterofermentative lactobacilli, including Latilactobacillus curvatus, Lpl. plantarum, Lcb. casei/paracasei, and obligate heterofermentative lactobacilli, such as Levilactobacillus brevis and Lentilactobacillus buchneri (Barbieri et al., 2019; Marcobal et al., 2012). All these species are common constituents of the ripening microbiota of several fermented foods. Strains of the same species can also decarboxylate histidine, even if this capability is less diffused among LAB (Barbieri et al., 2019; EFSA, 2011; Landete et al., 2008; Moniente et al., 2021). Putrescine derives from the decarboxylation of ornithine, an amino acid produced by the arginine deiminase pathway (ADI). This metabolic route is particularly advantageous because it allows the production of ATP from arginine. For this reason, ADI is advantageous for microorganisms in meat environment, in which fermentable sugar are rapidly depleted (Rimaux et al., 2012). However, in some strains, ornithine can be decarboxylate producing putrescine. This ability is rare in Lat. sakei (Barbieri et al., 2020), but it is more diffused among other species. Few studies concerning the ability of producing BA by C. alimentarius are available. One strain (out of two) belonging to this species was described as a relevant histamine producer by Straub et al. (1995). In addition, a strain of C. alimentarius isolated from meat was able to produce cadaverine, putrescine and tyramine (Min et al., 2004) and a strain from table olives presented a tyrosine decarboxylase activity (Yalçınkaya and Bas¸yigit ˘ Kılıç, 2019). By contrast, no decarboxylase activity was observed in strains isolated from Himalayan fermented foods (Dewan and Tamang, 2006, 2007).

Strains	Tyramine	Histamine	Putrescine	Cadaverine	2-phenylethylamine
CB1	15.1 ± 2.3	147.0 ± 21.9	ND*	ND	ND
CB6	61.5 ± 4.4	ND	132.8 ± 26.1	ND	ND
CB8	386.1 ± 16.5	ND	ND	ND	ND
CB16	489.2 ± 28.0	647.8 ± 69.1	271.1 ± 34.3	ND	ND
CB22	ND	ND	ND	ND	ND
CB31	ND	ND	ND	ND	ND
CB36	1156.6 ± 94.7	ND	ND	ND	ND
CB41	395.9 ± 45.2	ND	ND	ND	ND
CB43	ND	ND	ND	ND	ND
CE49	745.0 ± 78.5	90.8 ± 22.1	ND	ND	ND
CO12	30.1 ± 2.0	ND	ND	ND	ND
CO24	27.0 ± 1.4	ND	ND	ND	ND
CO50	ND	ND	ND	ND	ND
SE14	ND	641.0 ± 71.6	ND	ND	ND

Table 4*. Biogenic amines production by Companilactobacillus alimentarius strains. The data are the mean values of three repetitions and are expressed as mg/l with the relative standard deviation.*

**ND: not detected (under the detection limit: 3 mg/l).*

4.5. Growth kinetics in relation to temperature and NaCl concentration

Growth at different NaCl concentrations (0, 2.5 and 5 %) and temperatures (10, 20 and 30 ◦C) was monitored through the increase of OD600. The data were fitted with the Gompertz equation as modified by Zwietering et al. (1990) to estimate the parameter A, μmax and λ. The parameter estimated for each strain and condition and goodness-of-fit diagnostic of the regression are reported in Table S1. Fig. 1 shows the Box and Whisker plots describing the distribution of the three parameters in relation to temperature.

The median A value had no significant differences at 30 ◦C and 20 ◦C (2.02 and 2.05, respectively), while the μmax and λ medians were strongly affected by temperature. In particular, the value of μmax decreased from 0.331 to 0.186 and 0.042 OD600 h− 1 passing from 30 to 10 ◦C and the λ median value increased from 8.50 to 14.77 and 47.63 h under the same conditions. At 10 ◦C one of the strains did not grow (CB31). As it is possible to observe from Fig. 1, the strains presented, at the temperatures considered, a double behaviour. At 30 ◦C, a first group characterized by higher growth performances, both in terms of μmax and λ, included the strains CB6, CB16, CB36, CE49, CO50 and SE14. The same strains were responsible also for the best growth performances at 20 ◦C and 10 ◦C.

Concerning the effects of NaCl, the distribution of the estimated parameters is reported in the Box and Whiskers plots of Fig. 2. Increasing salt concentrations were responsible for the decrease of A (from a median value of 2.05 at 0 % to 1.74 at 5 %) and relevant μmax decreases (0.186 OD600 h− 1 at 0 % and 0.109 and 0.047 OD600 h− 1 at 2.5 and 5 %, respectively). The estimation of the λ median value slightly increased from 0 to 2.5 % (from 14.77 to 17.35 h) and more drastically at 5 % (26.43 h).

As already observed for the distribution of the parameters in relation to temperature, also in the case of the effect of NaCl on A, the performances of the strains can be clustered into two groups, one of which was characterized by lower final A values. This trend was particularly evident in the samples containing the higher salt concentration (CB1, CB8, CB31, CB41, CO12 and CO24). Concerning μmax the grouping effect attributable to NaCl was not evident, while it was found again in the distribution of λ. However, it is important to note that the stains showing lower A values did not coincide with the strains with longer λ estimates (CB6, CB16, CB36, CB43 and CE49).

Regarding pH, in Fig. 3 the Box and Whisker plots describing the parameters distribution in relation to temperature are showed, while all the data are reported in Table S2. No significant difference were observed in the pH median value decrease at 30 ◦C and 20 ◦C (approx. -2.10 units in both cases), among which CB43 and CO24 presented the more relevant pH decrease, while at 10 ◦C the median ApH value was lower (− 1.89). Significant differences were observed in μpHmax parameter, whose median value strongly decreased from 30 to 10 ◦C (− 0.193, − 0.100 and − 0.030 pH h− 1), with high variability at 30 ◦C (CV 34 %). The λpH estimate parameters are almost completely superimposable with the same parameter obtained measuring OD600, as demonstrated by the linear regression between the results obtained with the two methods (λpH = − 0.799 + 0.950·λOD600, R = 0.999).

Concerning the effect of NaCl, k value and the estimated parameters were significantly influenced by its concentration (Fig. 4 and Table S2). The more relevant differences were observed in μpHmax parameter, with the strains CB1, CB22, CB31, CB43, CE49 and CO24 presenting the lower values, especially at 0 and 2.5 % NaCl. Finally, as in the case of temperature effects, also the λpH estimated for pH were related with those determined with OD600 (λpH = − 1.037 + 0.953·λOD600, R = 0.959).

C. alimentarius is described as an environmental LAB growing between 15 and 37 ◦C, but information regarding its behaviour is scarce (Zheng et al., 2020). This study revealed its ability to growth at 10 ◦C (except for one strain) and a relatively high difference observed in the growth dynamics at the considered temperatures. Noteworthy, the growth at 10 ◦C can explain its presence in sausages in which low temperature can be applied during ripening. The adaptation to fermented meat environment is confirmed also by the performances showed in the presence of salt concentrations compatible with those characterizing sausages during manufacture and ripening.

5. CONCLUSIONS

C. alimentarius is a LAB species often found in fermented foods. In this paper, 14 strains, previously isolated from Spanish spontaneously fermented sausages, have been characterized for their technological and safety features.

In general, a wide variability was observed in relation to the parameters considered. Among phenotypic characters, glucose was the only carbon source used by all the strains, while the utilization of other carbohydrates is a strain-dependent characteristic. Regarding safety issues, several of the tested strains were able to produce histamine, tyramine and putrescine and only one was able to express all these

decarboxylating activities in relevant amounts. In addition, the antibiotic resistance greatly varied according to the strains, with the exception of vancomycin, to which all were resistant.

Concerning the technological parameters, C. alimentarius strains showed a relevant potential to grow in conditions of salt and temperature mimicking the level of this variables characterizing fermented sausages. Interestingly, the strains seem to show two different growth patterns, one of which characterized by lower growth potential, especially in relation to temperature.

In other words, the variability of the performances of the strains, concerning safety and technological parameters, reflects the differentiation of the phenotypic profile induced by the environmental conditions charactering the isolation source, including those derived by the raw materials.

Ultimately, among the 14 strains tested only 2 (CB22 and CB31) did not shown either decarboxylase activity or antibiotic resistance and could be candidate for a possible use as a starter culture. However, the strain CB31 was the only not able to growth at 10 °C and this could be a limitation for its use in product in which the ripening condition include low temperatures. Further studies are needed to better exploit the potential of these strains including, in particular, their contribution in the accumulation of compounds affecting the aroma profile of sausages.

Given the frequent association of this species with spontaneous fermentations or ripening microbiota of various products the data presented in this study contribute to a deeper comprehension of their role, both advantageous and detrimental, in fermented foods, even in vivo trials are necessary with this purpose.

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7. SUPPLEMENTARY MATERIALS

	0% NaCl			2.5% NaCl			5% NaCl			10°C			20°C			30°C		
Strains	*A*	μ_{max}	λ	*A*	μ_{max}	λ	*A*	μ_{max}	λ	*A*	μ_{max}	λ	*A*	μ_{max}	λ	*A*	μ_{max}	λ
CB1	1.83	0.090	22.43	1.75	0.052	20.11	1.50	0.026	23.40	1.78	0.016	238.45	1.83	0.090	22.43	1.93	0.222	11.57
CB6	2.08	0.286	15.21	2.07	0.105	15.13	1.83	0.049	37.11	1.83	0.042	46.92	2.08	0.286	15.21	2.06	0.413	9.54
CB8	2.02	0.221	15.20	1.97	0.171	17.48	1.59	0.054	27.72	1.76	0.031	45.55	2.02	0.221	15.20	2.01	0.306	7.46
CB16	2.08	0.216	13.79	2.04	0.126	17.23	1.73	0.046	33.74	1.98	0.042	47.63	2.08	0.216	13.79	1.99	0.419	8.59
CB22	2.00	0.170	14.33	1.81	0.109	20.35	1.78	0.043	21.29	1.70	0.032	54.86	2.00	0.170	14.33	1.97	0.310	7.25
CB31	1.90	0.087	18.50	1.83	0.091	18.24	1.33	0.042	23.82	-*	-	-	1.90	0.087	18.50	2.02	0.330	8.12
CB36	2.08	0.209	13.61	2.04	0.115	16.33	1.76	0.043	33.42	1.99	0.042	46.43	2.08	0.209	13.61	2.00	0.398	8.47
CB41	2.00	0.206	14.20	1.99	0.156	16.28	1.59	0.052	27.44	1.80	0.030	48.56	2.00	0.206	14.20	2.01	0.331	8.07
CB43	1.88	0.103	28.73	1.78	0.092	28.37	1.81	0.033	38.07	1.88	0.032	363.89	1.88	0.103	28.73	2.07	0.248	12.16
CE49	2.09	0.193	14.01	2.02	0.108	16.32	1.78	0.042	33.55	2.01	0.045	44.11	2.09	0.193	14.01	1.98	0.407	8.53
CO12	2.10	0.100	20.12	2.03	0.091	20.94	1.21	0.048	24.05	1.97	0.057	89.87	2.10	0.100	20.12	2.05	0.246	10.82
CO24	2.02	0.087	19.12	1.97	0.084	19.39	1.59	0.067	25.43	1.75	0.016	157.32	2.02	0.087	19.12	2.17	0.194	9.91
CO50	2.14	0.209	12.62	2.05	0.157	13.79	1.85	0.087	21.48	2.05	0.052	37.98	2.14	0.209	12.62	2.17	0.384	8.29
SE14	2.17	0.178	13.99	2.05	0.139	14.01	1.85	0.090	22.24	1.98	0.054	38.73	2.17	0.178	13.99	2.17	0.349	8.17
Median	2.05	0.186	14.77	2.00	0.109	17.35	1.74	0.047	26.43	1.88	0.042	47.63	2.05	0.186	14.77	2.02	0.331	8.50
Mean value	2.03	0.168	16.85	1.96	0.114	18.14	1.66	0.052	28.05	1.89	0.038	96.95	2.03	0.168	16.85	2.04	0.326	9.07
SD	0.10	0.064	4.50	0.11	0.033	3.71	0.20	0.018	5.95	0.12	0.013	99.53	0.10	0.064	4.50	0.08	0.075	1.52
CV	4.92	37.82	26.73	5.80	28.92	20.47	12.06	35.43	21.19	6.17	34.51	102.67	4.92	37.82	26.73	3.78	23.07	16.72

Table S1. *Gompertz parameters (A, μmax and λ) of C. alimentarius strains growth at 20°C in presence of different salt concentrations (0, 2.5 and 5%) and at different incubation temperatures (10, 20 and 30°C) in absence of salt. Median, mean value, standard deviation (SD) and variability coefficient (CV) are also reported.*
**: no growth was observed*

	0% NaCl				2.5% NaCl				5% NaCl				10°C				20°C				30°C			
Strains	k	A_{pH}	μ_{pHmax}	λ_{pH}	k	A_{pH}	μ_{pHmax}	λ_{pH}	k	A_{pH}	μ_{pHmax}	λ_{pH}	k	A_{pH}	μ_{pHmax}	λ_{pH}	k	A_{pH}	μ_{pHmax}	λ_{pH}	k	A_{pH}	μ_{pHmax}	λ_{pH}
CB1	6.28	-2.13	-0.084	21.20	6.09	-2.10	-0.054	20.55	6.01	-1.99	-0.035	25.38	6.32	-1.93	-0.018	230.54	6.28	-2.13	-0.084	21.20	6.33	-2.11	-0.178	10.75
CB6	6.22	-2.07	-0.105	10.76	6.04	-1.96	-0.101	16.86	6.03	-1.96	-0.039	30.03	6.01	-1.53	-0.035	54.17	6.22	-2.07	-0.105	10.76	6.25	-2.11	-0.208	8.41
CB8	6.22	-2.06	-0.110	12.96	6.05	-2.05	-0.091	14.89	6.02	-1.90	-0.050	24.64	6.25	-1.90	-0.030	39.98	6.22	-2.06	-0.110	12.96	6.25	-2.06	-0.203	6.77
CB16	6.33	-2.15	-0.099	10.62	6.10	-2.01	-0.072	14.74	5.99	-1.87	-0.037	30.80	6.35	-1.91	-0.034	48.73	6.33	-2.15	-0.099	10.62	6.34	-2.08	-0.314	8.34
CB22	6.22	-2.05	-0.066	10.23	6.03	-2.00	-0.055	13.46	5.96	-2.10	-0.034	21.46	6.22	-1.76	-0.020	48.25	6.22	-2.05	-0.066	10.23	6.25	-2.18	-0.105	7.27
CB31	6.18	-2.08	-0.074	19.38	6.16	-2.05	-0.049	15.80	5.95	-1.96	-0.042	22.50	-*	-	-	-	6.18	-2.08	-0.074	19.38	6.18	-1.91	-0.120	7.30
CB36	6.34	-2.15	-0.109	11.18	6.11	-2.00	-0.074	14.36	6.00	-1.83	-0.041	32.76	6.35	-2.04	-0.033	45.56	6.34	-2.15	-0.109	11.18	6.31	-2.04	-0.332	8.51
CB41	6.23	-2.07	-0.110	12.80	6.06	-2.06	-0.089	14.16	6.01	-1.94	-0.050	25.09	6.24	-1.98	-0.030	43.00	6.23	-2.07	-0.110	12.80	6.26	-2.07	-0.214	7.38
CB43	6.32	-2.41	-0.065	22.62	6.09	-2.24	-0.058	23.48	5.98	-2.11	-0.038	37.06	6.30	-2.03	-0.023	345.57	6.32	-2.41	-0.065	22.62	6.34	-2.34	-0.183	10.56
CE49	6.36	-2.18	-0.094	10.63	6.13	-2.07	-0.061	13.31	5.99	-1.86	-0.038	32.04	6.35	-2.05	-0.033	42.86	6.36	-2.18	-0.094	10.63	6.30	-2.04	-0.280	8.15
CO12	6.18	-2.12	-0.100	19.85	6.14	-2.11	-0.098	19.79	6.04	-1.80	-0.053	23.21	6.21	-1.77	-0.037	85.72	6.18	-2.12	-0.100	19.85	6.29	-2.30	-0.150	8.76
CO24	6.26	-2.39	-0.072	17.50	6.07	-2.27	-0.066	18.70	6.00	-2.20	-0.049	22.84	6.22	-1.91	-0.015	136.64	6.26	-2.39	-0.072	17.50	6.31	-2.48	-0.133	7.99
CO50	6.23	-2.14	-0.109	10.68	6.05	-1.99	-0.096	13.43	6.04	-1.83	-0.062	20.36	6.12	-1.75	-0.023	31.49	6.23	-2.14	-0.109	10.68	6.24	-2.17	-0.180	6.27
SE14	6.19	-2.12	-0.108	11.69	6.03	-1.96	-0.093	12.73	6.03	-1.77	-0.066	20.98	6.19	-1.65	-0.028	40.03	6.19	-2.12	-0.108	11.69	6.22	-2.15	-0.223	7.76
Median	6.23	-2.13	-0.100	12.25	6.08	-2.05	-0.073	14.81	6.01	-1.92	-0.042	24.86	6.24	-1.91	-0.030	48.25	6.23	-2.13	-0.100	12.25	6.27	-2.11	-0.193	8.07
Mean value	6.25	-2.15	-0.093	14.43	6.08	-2.06	-0.075	16.16	6.00	-1.94	-0.045	26.37	6.24	-1.86	-0.028	91.73	6.25	-2.15	-0.093	14.43	6.28	-2.15	-0.202	8.16
SD	0.06	0.11	0.017	4.58	0.04	0.09	0.019	3.27	0.03	0.13	0.010	5.21	0.10	0.16	0.007	94.33	0.06	0.11	0.017	4.58	0.05	0.14	0.069	1.27
CV	0.98	5.16	18.67	31.75	0.71	4.55	24.72	20.21	0.46	6.53	22.01	19.75	1.61	8.64	25.64	102.83	0.98	5.16	18.67	31.75	0.80	6.75	34.02	15.53

Table S2. Gompertz parameters (*A*, *μmax* and *λ*) of *C. alimentarius* strains pH decrease at 20°C in presence of different salt concentrations (0, 2.5 and 5%) and at different
incubation temperatures (10, 20 and 30°C) in absence of salt. Median, mean value, standard deviation (SD) and variability coefficient (CV) are also reported.

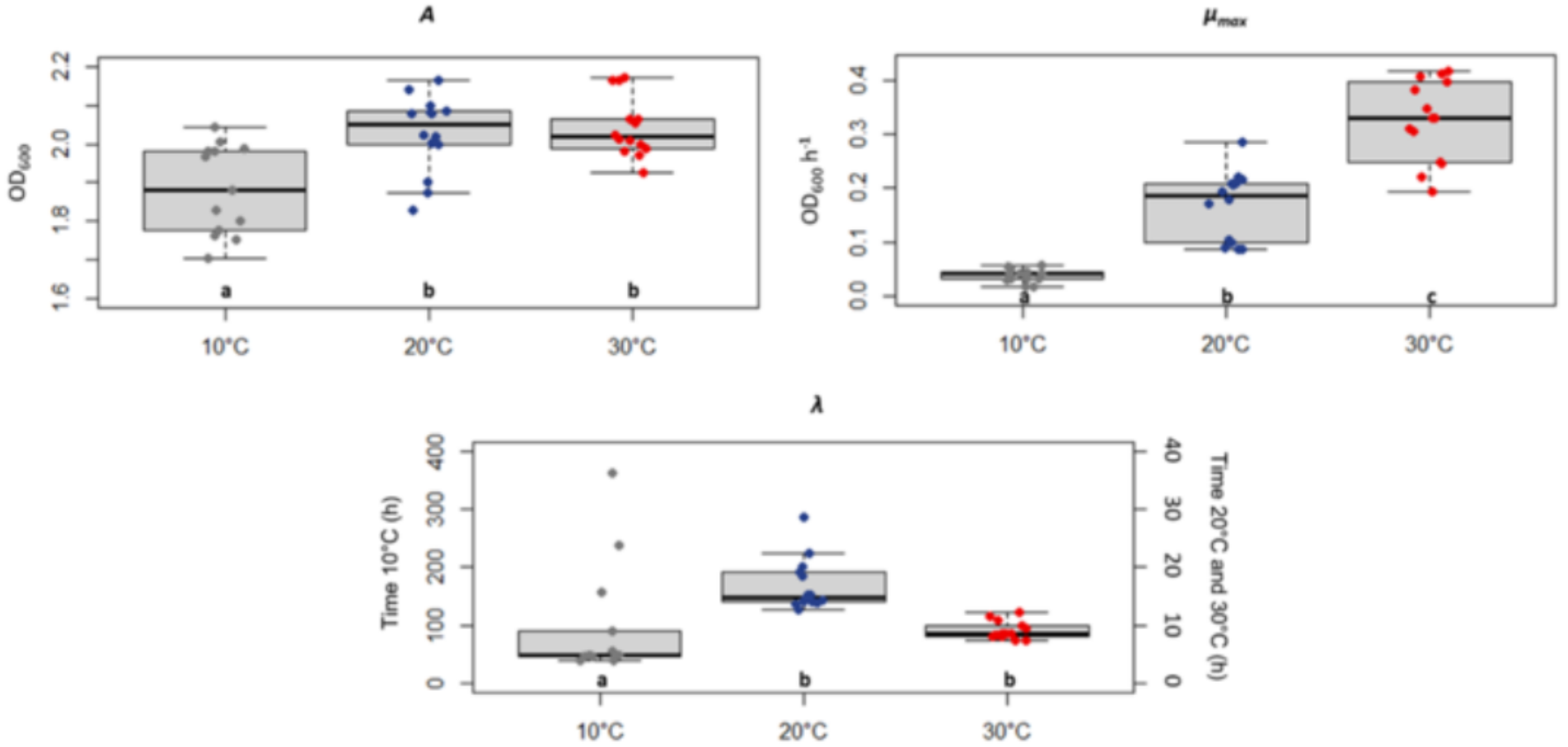

Figure 1. *Box and Whisker plots representing the distribution of parameters estimated by Gompertz equation (A, μmax and λ) of strain growth kinetics at different incubation temperatures (10, 20 and 30°C). In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from*
median exceeds at least ± 1.5 times the box height. Different letters indicate significant differences ($p \leq 0.05$) according to ANOVA.

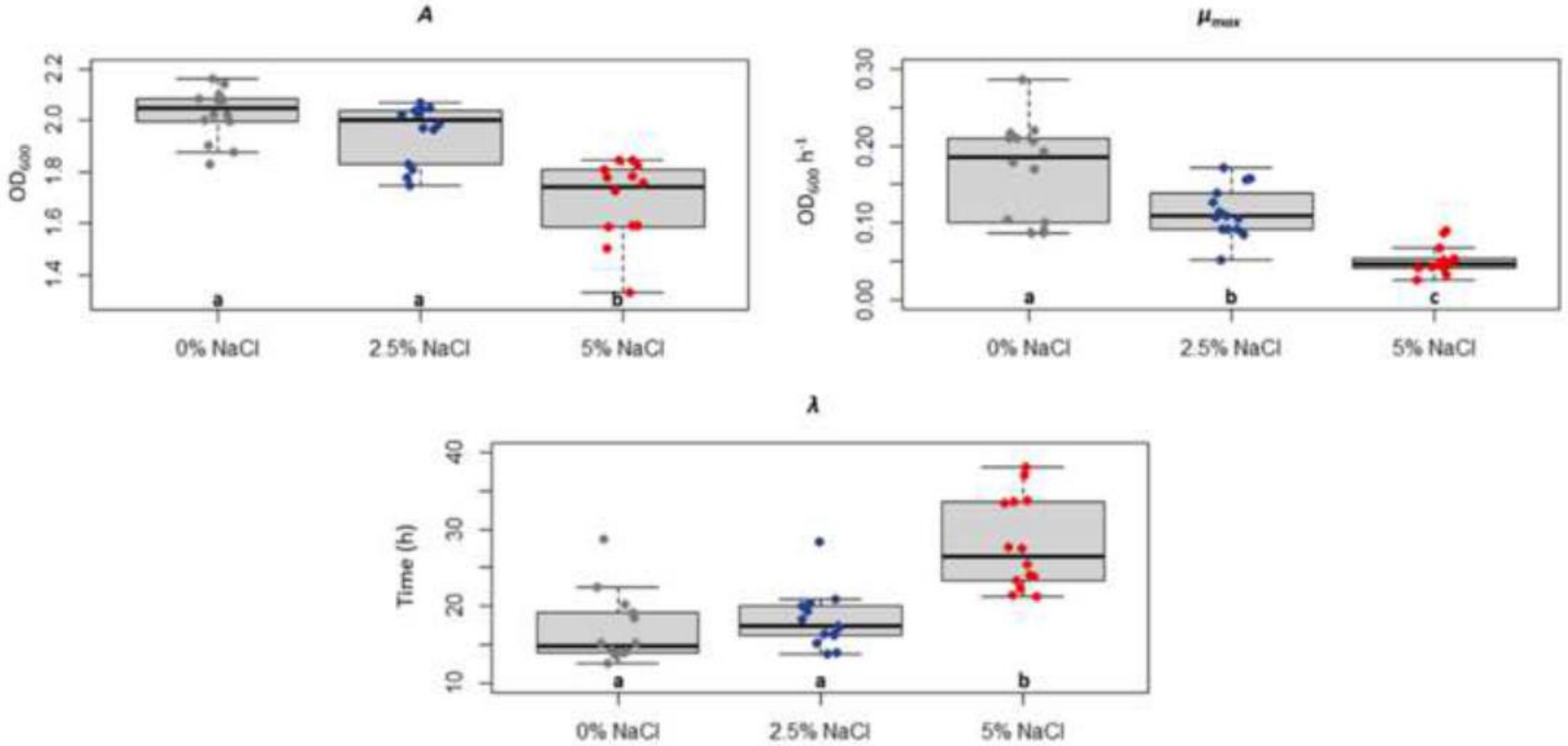

Figure 2. *Box and Whisker plots representing the distribution of parameters estimated by Gompertz equation (A, μ_{max} and λ) of strain growth kinetics at different salt concentrations (0, 2.5 and 5%). In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ± 1.5 times the box height. Different letters indicate significant differences ($p \leq 0.05$) according to ANOVA.*

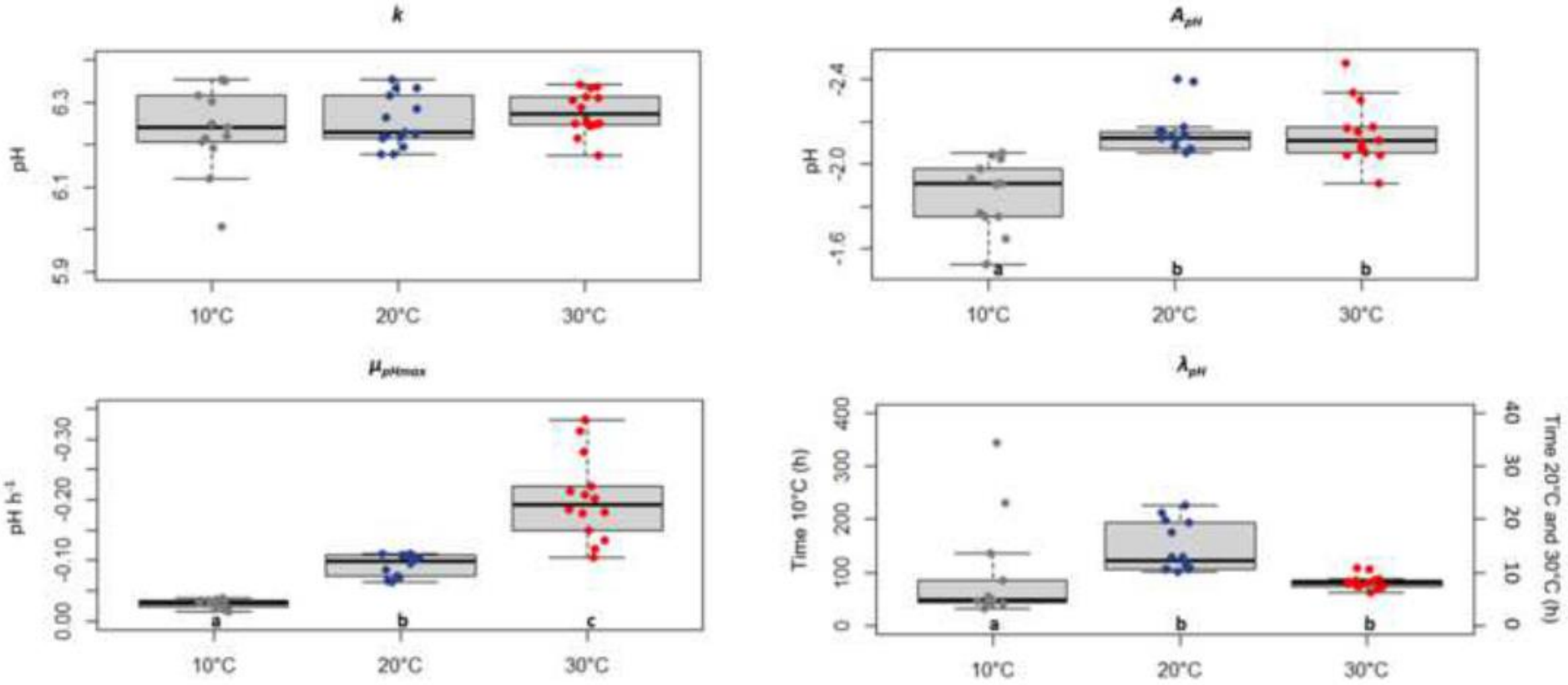

Figure 3. *Box and Whisker plots representing the distribution of parameters estimated by Gompertz equation (k, ApH, μpHmax and λpH) of pH decrease at different incubation temperatures (10, 20 and 30°C). In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ± 1.5 times the box height. Different letters indicate significant differences ($p \leq 0.05$) according to ANOVA.*

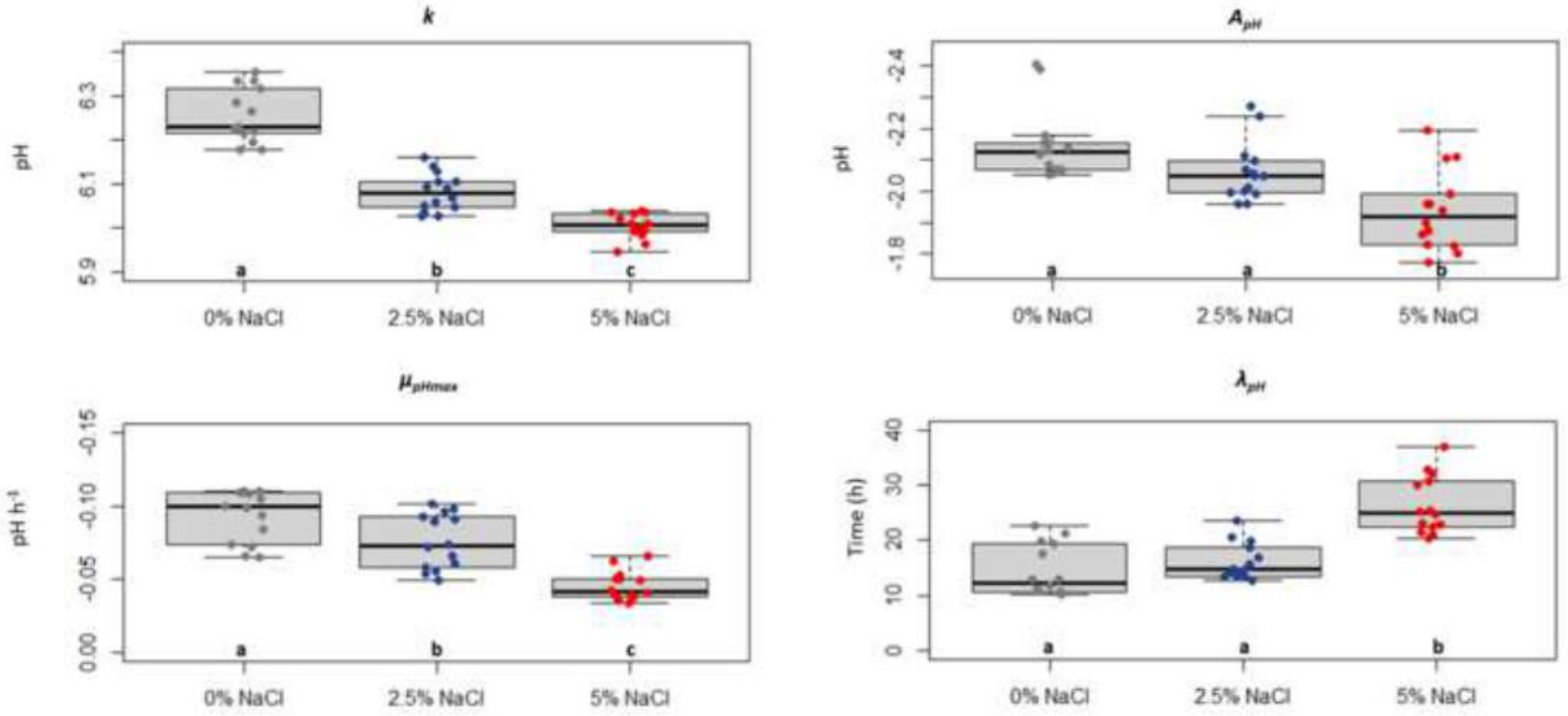

Figure 4. *Box and Whisker plots representing the distribution of parameters estimated by Gompertz equation (k, ApH, μpHmax and λpH) of pH decrease at different salt concentrations (0, 2.5 and 5%). In the boxes the thick line represents the median value, the limit of the boxes is 25th and 75th percentile and the two whiskers are the minimum and maximum values, excluding outliers. Outliers are defined as points whose distance from median exceeds at least ± 1.5 times the box height. Different letters indicate significant differences ($p \leq 0.05$) according to ANOVA.*

CHAPTER 9
General Conclusions

Ensuring the quality and safety of food is of paramount importance, serving the dual purpose of safeguarding consumer health and bolstering the food industry's integrity. Therefore, it becomes imperative to rapidly and sensitively monitor the presence of harmful microbes that could enter the food supply chain, potentially contaminating raw materials or food products at various processing stages. Technological advancements have provided novel molecular analyses tecniques, greatly enhancing the detection and characterization of microorganisms in food. These modern techniques complement traditional microbiological methods, offering improved efficiency and precision.

However, molecular methods also have limitations, as they usually focus on one or a few characteristics of a microorganism and can be compromised by technical issues such as PCR inhibitors present in the food matrix. In recent years, WGS has expanded the potential of molecular methods by providing the possibility of in-depth analysis of individual micorbes.

WGS provides unparalleled discriminatory power in bacterial identification and characterization, making it superior to any other technique. Consequently, the choice between a rapid molecular method and the exhaustive capabilities of WGS depends on the specific objectives of the study.

This study initially focused on the assessment of AMR and pathogenicity potential of bacteria isolated from food and animal sources, using WGS approach. Subsequently, the transferability of ARG was studied using a combination of *in vitro* assays, food models, *in vivo* models, and environmental models. Following this, specific virulence factors important in the pathogenesis process in *Galleria mellonella* larvae were investigated. Additionally, the ability of *E. coli* STEC to persist and survive the food transformation process was investigated, replicating real-world conditions on a laboratory scale. Finally, our attention turned to the application of LAB, sourced from spontaneously fermented food matrices as potential starter cultures and/or bioprotective agents.

Chapters two and three mainly focused on the critical aspects of surveillance and early detection of foodborne AMR bacteria, including taxonomic identification, pathogenic potential and antimicrobial resistance, which are addressed by the WGS approach combined to traditional microbiological and molecular methods. A concern faced by food sector globally revolves around the prevalence of AMR bacteria. Curtailing the use of antimicrobials is a shared responsibility, spanning both the human and animal sectors, intrinsically intertwined with the environment within the framework of "One Health". The presence of MDR strains in ready-to-eat fermented foods poses a public health risk, contributing to

the dissemination of AMR determinants within the food chain and in the gastrointestinal microbiota of consumers. For example, the application of in silico bioinformatics has proven instrumental in assessing the safety profile of the UC7251 strain of *E. faecium*. Moreover, the spread of AMR across ecosystems has facilitated its entry into the food chain. The widespread nature of AMR underscores the importance of obtaining comprehensive information on the reservoirs of resistance.

From this perspective, the circulation of EsβLs-producing *E. coli* strains raises notable concerns, given their resistance to second and third generation cephalosporins, thereby limiting the availability of alternative medical treatments. The investigation, underpinned by phenotypic, molecular, and genomic characterization of ESβLs-producing *E. coli* strains isolated from wild boars, has revealed their dual role as ESβLs producers and MDR entities. Additionally, genome analysis has played a pivotal role in elucidating the virulence potential and mobilome of ESβLs-producing *E. coli*, ultimately leading to the identification of two pandemic serotypes. As previously mentioned, the ability to thoroughly characterize AMR strains has substantially enriched our understanding of AMR reservoirs and of the environmental spread of these strains, providing insights into the circulation of problematic *E. coli* clones. In this context, future studies investigating strains circulating in the same environment, such as farm animals or other wildlife, will provide valuable information on the AMR transmission dynamics.

The fourth chapter focused on assessing the capability of MDR *E. faecium,* isolated from fermented meat, to transmit tetracycline resistance to two unrelated strains of the foodborne pathogen *L. monocytogenes*. This study on gene transfer covered a range of conditions, starting with traditional *in vitro* analyses and extending to more intricate models. Notably, the transfer of genetic material was examined in sausage and cheese models, effectively simulating the conditions prevalent within these food matrices. Subsequently, *in vivo* gene exchange was investigated through the infection of larvae, providing a representation of scenarios found within the gastrointestinal tract of mammals. Lastly, the study explored gene transfer within a complex marine ecosystem characterized by the presence of *Mytilus galloprovincialis* (Mediterranean mussel) and microplastics. The study revealed significant rates of gene transfer across different ecological settings and within food-related scenarios. Models have been designed to evaluate HGT among commensal and foodborne pathogenic bacteria, thereby improving our understanding of these dynamics in the food system. Furthermore, the study underscored the role of environmental pollutants, such as microplastics in marine bivalves, in promoting the spread of transposon-encoded AMR among food-associated bacteria.

The fifth chapter was dedicated to the assessment of the pathogenic potential of *E. coli* STEC strains isolated from a semi-matured raw milk cheese. The plasticity of the *E. coli* genome suggests a high variability between strains, which translates into a high capacity for the exchange of genetic material, including pathogenicity islands and plasmids. Genome analysis provided insights into the pathogenicity of Shiga toxin *E. coli* STEC, which is the fourth leading cause of foodborne illness. The findings highlighted that the strain harbours not only a rich collection of virulence factors, but also a recently

described pathogenicity island discovered in emerging STEC subtypes. Furthermore, this chapter explored the pathogenicity and persistence of *E. coli* STEC mutants. lacking one or both Shiga toxins. Their behaviour was assessed using *Galleria* larvae as a model for *in vivo* pathogenicity, and within a simulated cheesemaking environment. The outcomes demonstrated that *in vivo* pathogenicity cannot be exclusively attributed to Shiga toxins and that the raw milk cheese production process alone is insufficient to prevent the growth and survival of STEC in the final product.

Finally, chapters six, seven and eight focused on isolation and characterisation of potential novel starter and bioprotective LAB strains from naturally fermented sausages. By sampling 15 natural fermented sausages, 151 different LAB biotypes were isolated, including strains belonging to the species *L. sakei*, *L. curvatus* and *C. alimentarius*. After technological and safety characterisation, the strains found to be safe and therefore suitable for use in food were tested *in vitro* and in sausage models to assess their inhibitory action against pathogenic bacteria such as *E. coli*, *L. monocytogebens* and *Salmonella* Enteritidis. Several strains of LAB demonstrated favourable technological attributes and antimicrobial potential. These strains hold significant promise for the use as starter cultures or bio-preservative agents in meat-based food systems, such as fresh products and fermented sausages, to enhance the quality of food products, ensure microbiological safety, and provide distinctive sensory characteristics in the final product.

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