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ARTISANAL FOOD PRODUCTIONS OF ANIMAL ORIGIN: EXPLORING FOOD SAFETY IN THE AGE OF WHOLE GENOME SEQUENCING

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"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world."

-Louis Pasteur

Abstract

The artisanal food chain is enriched by a wide diversity of local food productions with delightful organoleptic characteristics and valuable nutritional properties. Despite their increasing worldwide popularity and appeal, several food safety challenges are addressed in artisanal facilities context suffering from less standardized processing conditions. In such scenario, recent advances in molecular typing and genomic surveillance (e.g., Whole Genome Sequencing [WGS]) represent an unprecedent solution capable of inferring sources of contamination as well as contributing to food safety along the artisanal food continuum.

The overall objective of this PhD thesis was to explore potential microbial hazards among different artisanal food productions of animal origins (dairy and meat-derived) typical of the food culture and heritage landscape belonging to Mediterranean countries. Three different studies were then carried out, specifically focussing on: 1) compare the seasonal variability of microbiological quality and potential occurrence of microbial hazards in two batches of Italian artisanal fermented dairy and meat productions; 2) Investigate genetic relationships as well as virulome and resistome of foodborne pathogens isolated within dairy and meat-derived productions located in Italy, Spain, Portugal and Morocco; 3) investigate the population structure, virulome, resistome and mobilome of *Klebsiella* spp. isolates collected from study 1, including an extended range of public sequences.

Study 1: ISO standards along with biochemical and molecular identification tests have been applied to investigate the microbiological quality of two artisanal fermented dairy and meat productions from Northern Italy (soft cheese from pasteurized cow milk and pork meat salami). A total of 250 and 140 samples respectively, were collected from processing environments, raw materials, semi-finished and final products (cheese and salami) plus during product shelf-life (cheese) from two batches produced in summer and winter between 2020 and 2021. The cheese production was affected by significant seasonal variability, showing in winter a boost of total bacterial count (TBC ≥7.71 $\log_{10}$ CFU/g) and lactic acid bacteria (LAB ≥3.94 $\log_{10}$ CFU/g) corresponding to the final storage phase. Moreover, strains of *S. aureus* and *K. oxytoca* were disseminated through environmental sites, semi-finished and final cheese product, suggesting that the worsened microbiological and physicochemical (aw ≥0.99; pH ≥5.3) conditions of the winter batch, may have promoted their persistence via cross-contaminations. On the other hand, the natural evolution of indigenous microbial consortia over 6 months of ripening in salami has led to TBC and LAB increase with higher load in summer (TBC ≥8.77 $\log_{10}$ CFU/g; LAB ≥7.71 $\log_{10}$ CFU/g), alongside a reduction of *Enterobacteriaceae* (ENT ≤1.09 $\log_{10}$ CFU/g) and water activity (Aw ≤0.88). Whilst *S. aureus* and *K. pneumoniae* strains contaminated raw materials, semi-finished salami and environmental sites, the total absence of foodborne pathogens in final products suggests the 6-month ripening as an effective hurdle.

Study 2: Whole genome sequencing approaches were applied on genomes of foodborne pathogens isolated from over 2,800 samples overall collected from raw materials, semi-finished, final products and environmental sites in 4 to 6 batches of each food production between 2019-2021, 42 isolates were confirmed as *L. monocytogenes* (n=14), *S. enterica* (n=13), and *S. aureus* (n=15). High-

resolution typing and MLST/SNP-based phylogeny confirmed that different clones circulated in the same artisanal production facilities over several months. Several clones harboured virulence or hypervirulence as well as antimicrobial resistance (AMR) related genes. *L. monocytogenes* ST1 and ST8 carried several pathogenic features, such as the full-length *inlA* gene, the Listeria Pathogenicity Island 1 (LIPI1) and 3 (LIPI3). These isolates persisted from the environment to fermented sausage of Spanish artisanal plant. One clone of *S.* Paratyphi B ST43 persisted over one year in the Portuguese meat processing environment to final products and exhibited a high virulence repertoire (i.e., pathogenicity islands SP1, SP2, SP3). Diverse *S. aureus* genotypes were introduced in artisanal meat and cheese facilities of Spain, Italy and Morocco over time as a result of different cross-contamination events, with ST121 harbouring the highest pathogenic potential. Concerning AMR features, many genes encoding for aminoglycosides, beta-lactams, sulphonamides, trimethoprim and tetracyclines resistance were carried by *S. enterica* serovar Hadar and Senftenberg from Moroccan fermented sausages. Despite the overall low prevalence of bacterial foodborne pathogens detected across all productions, enhanced prevention and hygienic control measures are required to strengthening barriers to these biological hazards in the artisanal food chain and thus safeguard consumers health.

Study 3: Whole genome sequencing approaches were applied in order to: (i) assess the occurrence of *Klebsiella* spp. strains through the food chain of the selected productions and improve their taxonomic characterization, (ii) describe the distribution and spread of genetic features of proven clinical importance (i.e., AMR and virulence), (iii) investigate the population structure including an extended range of public sequences as well as evaluate strains relatedness among the selected productions. Given the presence of *Klebsiella* spp. reported in the previous study on the Italian artisanal facilities, a larger collection of samples, encompassing six cheese and salami batches respectively sampled over 14 months, were investigated to elucidate the role of *Klebsiella* spp. as potential foodborne pathogens. Over 1,170 samples were collected with a *Klebsiella* prevalence of (6%). Seventy-three strains were classified into *K. pneumoniae* (KpSC, n=17), *K. oxytoca* (KoSC, n=38) and *K. planticola* (KplaSC, n=18) species complex. Whilst high genetic diversity was highlighted in terms of known and new STs, core gene phylogeny revealed clonal strains persisting in the same processing setting for over weeks or months, and contaminating the environment, raw materials, and end-products both of the dairy and meat-derived production chain. A high genetic proximity (≤1.5% allele differences) was observed by cgMLST between 8 *K. pneumoniae* and 2 *K. oxytoca* food strains and human strains from public repositories suggesting possible transmission from food to human or vice versa. All genomes showed a relatively low number of chromosomally located AMR genes. Specifically, *K. pneumoniae* genomes showed the highest virulence potential, with sequence types ST4242 and ST107 strains carrying yersiniabactin *ybt16* and aerobactin *iuc3*. All *K. pneumoniae* from salami harboured indeed a large conjugative *iuc*3^+ plasmid similar (97% identity) to *iuc*3^+ plasmids from human and pig strains circulating in nearby regions, suggesting possible transfer of virulence plasmids across human, livestock and food. Overall, this study provides new evidence on the dissemination of virulence plasmids across a different ecological niche and emphasizes on the need of further monitoring *Klebsiella* spp. to better understand its role as potential foodborne microbiological hazard.

List of abbreviations

AB, Antibiotic

AMR, Antimicrobial Resistance

AMRG, Antimicrobial Resistance Genes

Aw, Water Activity

CFU, Colony Forming Unit

cgMLST, Core genome Multilocus Sequence Typing

CNS, coagulase-negative staphylococci

COVID-19, Coronavirus Disease 2019

EFSA, European Food Safety Authority

EU, European Union

ESBLs, Extended spectrum β-lactamases

FBOp, Food Business Operators

FWDs, Foodborne and Waterborne Diseases

HACCP, Hazard Analysis and Critical Control Point

HTS, High Throughput Sequencing

ISO, International Organization for Standardization

LAB, Lactic Acid Bacteria

LMICs, Low- and Middle-income Countries

MBLs, Metallo-β-lactamases

MDR, Multi-drug Resistant

MGE, Mobile Genetic Element

MLST, Multilocus Sequence Typing

MLVA, Multilocus Variable-number Tandem-repeat Analysis

MS, Member States

NGS, Next Generation Sequencing

PCR, Polymerase Chain Reaction

PFGE, Pulsed-field Gel Electrophoresis

RTE, Ready-to-eat

SNP, Single-nucleotide Polymorphism

ST, Sequence Type

STEC, Shiga toxin-producing *Escherichia coli*

VNTR, Variable-number Tandem Repeat

wgMLST, Whole genome Multilocus Sequence Typing

WGS, Whole Genome Sequencing

WHO, World Health Organisation

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Introduction

1. The global foodborne disease burden

1.1. Food safety surveillance within the frame of the European Union

The food supply chain represents a global system where producers, distributors, consumers and regulators play critical distinct roles but systematically interconnected through the farm to fork continuum. Among this articulated system, it becomes challenging to adapt its management with population growth, globalization and fluctuating consumers' behaviour. Some of these challenges are concerned with food safety hazards such as emerging foodborne pathogens, spoilage organisms, allergens, and other risks which could enter the food chain from production to consumption. Thus, a continuous active surveillance along the food supply chain is needed to actively identify, decipher and mitigate these risks, especially the microbiological hazards, in order to preserve population's health as well as food's quality (Imanian et al., 2022).

Bacteria, viruses, parasites or chemical substances such as heavy metals, can contaminate food at any stage of the food chain, including production, delivery and consumption. The consumption of contaminated food may cause foodborne diseases. A wide range of symptomatology is concerned with foodborne diseases, ranging from diarrhoea to cancers. The most common illnesses are of gastrointestinal diagnosis, but additional implications could be addressed with neurological, gynaecological or immunological symptoms. Either reporting mild or severe disorders, foodborne illnesses dramatically contribute to the global public health burden of disease and mortality, thus causing socioeconomic problems regarding lost productivity of health-care systems as well as harming tourism and trade (World Health Organization, 2022).

In general, foodborne diseases can occur as sporadic cases or outbreaks. A foodborne disease outbreak is characterized by two or more cases with a specific syndrome, such as vomiting or diarrhea, reported in a specific geographical region over a short period of time and linked to the same food source. When a foodborne outbreak occurs, epidemiological investigation represents a crucial phase aimed at identifying the causative agent as well as the suspected food vehicle (Hoelzer et al., 2018).

The first global estimation of foodborne illnesses impact was published by the World Health Organization (WHO) in 2015, which was set up on 2010 data. The estimation accounted about 600 million cases of foodborne illnesses and 420,000 associated deaths each year at the global scale. Moreover, the data suggested that 31 known pathogens were the leading causes of foodborne deaths, including (i) non-typhoidal *Salmonella* (approx. 59,000 deaths), (ii) *Salmonella Typhi* (approx. 52,000 deaths), (iii) Enteropathogenic *Escherichia coli* (about 37,000 deaths), and (iv) *Norovirus* (approx. 35,000 deaths) (Hoelzer et al., 2018).

Despite the high number of reported cases, these data are likely to represent a huge underestimation of the real number of foodborne illnesses occurring every year. This could be generated by effective cases of foodborne diseases which stay unnoticed or might be misinterpreted as individual sporadic cases not linked to one another. Situations in which outbreaks are geographically dispersed or concern small number of cases occurring over long periods of time are just few examples of outbreaks'

underestimation. Reporting of cases is not harmonized between counties, and only few countries have been supported by reliable and well-documented estimates of foodborne disease burdens. Even within a given country, the ability of individual foodborne disease surveillance systems to identify and record cases varies drastically among regions, states and administrative districts. For instance, many cases of diseases that generally show milder symptoms never reach a proper diagnosis. As a consequence, comparing foodborne disease burdens among countries and across time periods results in inaccurate outcomes and challenges policymakers. The presence of a well-developed food safety and infectious disease surveillance systems reduces the risk of underdiagnosed numbers of foodborne diseases (Scallan et al., 2011).

Among the European Union (EU), 10 out of 22 Member States declared that the Coronavirus Disease 2019 (COVID-19) pandemic impacted on their surveillance/monitoring systems of human cases of foodborne and waterborne diseases (FWDs) (brucellosis, campylobacteriosis, echinococcosis, listeriosis, salmonellosis, Shiga toxin-producing *Escherichia coli* (STEC) infection, trichinellosis, congenital toxoplasmosis and yersiniosis). Notably, a drop in the notification rates was highlighted for all zoonoses except trichinellosis and yersiniosis when comparing data from 2020 and 2019, showing a reduction from 52.6% to 7.1% depending on the zoonosis of concern. Several factors might have had an effect on surveillance activities and the reporting of FWDs data (European Food Safety Authority & European Centre for Disease Prevention and Control, 2021), namely national health care resilience (health work force, laboratory and diagnostic capability, access to hospitals and medical assistance), the shutdown of domestic and international travel, restrictions on sporting and recreational/social events, the closing of restaurants and catering facilities (e.g., schools, workplaces), quarantine, lockdown and other non-pharmaceutical mitigation measures (face masking, hand washing/sanitisation, physical distancing, restricted movement and social gatherings).

Regardless of the underdiagnosed cases, the WHO report highlighted the global impact of foodborne illnesses, which is even more worrisome in world's most vulnerable populations. The highest burden is estimated for two African sub-regions covering large parts of the African continent and the 40% of global illnesses are associated with children under 5 ages. Taken together, these data draw the global attention to the development of tools and resources that can be universally available to prevent foodborne illness cases and outbreaks (Hoelzer et al., 2018).

In December 2022, the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) published the report on 2021 surveillance data on zoonoses and zoonotic agents in humans, food, animals and feed for 27 EU Member States (MS), the United Kingdom (Northern Ireland) and nine non-MS. Here, EFSA was tasked to characterize the causal agents and foodstuffs implicated in foodborne outbreaks, as well as their temporal patterns. This characterization aimed at (i) investigating the impact of foodborne outbreaks on public health in Europe and (ii) pointing out the food manufacturing and distribution chains that are most involved (European Food Safety Authority & European Centre for Disease Prevention and Control, 2022).

In 2021, 27 EU Member States and the United Kingdom (Northern Ireland) reported 4,005 foodborne outbreaks, 32,543 cases of illness, 2,495 hospitalisations and 31 deaths, whereas 83 outbreaks, 1,270 cases of illness, 65 hospitalisations and 2 deaths were documented for seven non-MS.

In comparison to 2020, the number of reported outbreaks increased by 29.8% (3,086 in 2020), along with human cases and hospitalizations, which respectively increased by 62.6% (20,017 cases in 2020) and 49.0% (1,675 hospitalizations in 2020). Nevertheless, in comparison to most recent pre-pandemic years (2017–2019) the total number of outbreaks, cases and hospitalizations reported in 2021 decreased in average (28.5% for outbreaks, 34.2% for cases and 44.3% for hospitalizations).

Besides the increasing trend of prevalence of foodborne outbreaks, a significant influence of the COVID-19 pandemic on cases reporting is suggested in EU for 2021. Indeed, across the second year of the COVID-19 pandemic National EU authorities implemented several control measures to obstacle the virus's spread, including contact restriction measures (e.g., stay-at-home orders, the prohibition of private meetings, and so on), hygiene and safety precautions (e.g., the use of protective equipment, disinfection processes, and so on), and other restrictions. Although measures adopted in 2021 were less strict compared to 2020, they showed an impact in decreasing the foodborne exposure of the population to zoonotic agents (e.g. better hygiene, closure of restaurants, etc.), improving hygiene measures by consumers (e.g. gloves, hand sanitizer, cleaning of surfaces and equipment, etc.) or even in reducing travel-related FBOs, as observed in the literature (Ray, 2021; van Deursen et al., 2022). Moreover, the COVID-19 pandemic affected the healthcare services, leading to a weakened doctor-patient interaction, healthcare difficulties and under-diagnosis and under-reporting of non-COVID diseases (Kastritis et al., 2020; Lim et al., 2021; Verhoeven et al., 2020). Nonetheless, the increasing foodborne outbreaks number may suggest a progressive return to pre-pandemic surveillance stability for most Member States.

Among foodborne bacterial outbreaks, *Salmonella* remains the most frequently identified causative agent (19.3% of total outbreaks, n=773), being responsible for the highest number of confirmed cases (n=6,755) and hospitalisations (n=1,123) (Figure 1). Looking at *Salmonella* serovars, *S.* Enteritidis was the most prevalent (n=350; 79.7%), followed by *S.* Typhimurium (n=50; 11.4%), *S.* Braenderup (n=9; 2.1%) and *S.* Typhimurium monophasic (N = 6; 1.4%). Moreover, *Salmonella* represented the main cause of foodborne outbreaks in most EU Member States (n=17) and the United Kingdom (Northern Ireland), as well as in six non-Member States (European Food Safety Authority & European Centre for Disease Prevention and Control, 2022).

According to the EFSA summary report, *L. monocytogenes* was still confirmed responsible of the highest numbers of deaths among outbreak cases and case fatality rates (Figure 1). Moreover, in 2021 the number of listeriosis outbreaks was higher than in both 2020 and the pre-pandemic years. Notably, since outbreaks involved fewer cases, hospitalisations and deaths, an increased detection of small listeriosis outbreaks in the population is suggested.

The third most frequently cause of foodborne outbreaks was STEC (Figure 1), which overall showed a decreasing trend in both outbreaks and illness number over the past 4 years (from 50 to 31 across the period 2018-2021). Among STEC outbreaks, the most frequently serogroups resulted O157 (n=9), O26 (n=6), O103 (n=5) and O12, O145, O146, O91 (n=1 each). Other than STEC, Enteroinvasive *E. coli* (EIEC) and Enterotoxigenic *E. coli* (ETEC) were reported in five and two foodborne outbreaks respectively.

Campylobacter was responsible of n=112 outbreaks in Member States, with *C. jejuni* (n=106 outbreaks) and *C. coli* (n=6 outbreaks) as the most prevalent species. Although *Campylobacter* outbreaks are generally characterized by relatively mild illnesses, in 2021 it has been observed the highest number of deaths since 2007, thus raising the need to increased effort on monitoring.
Moreover, 679 foodborne outbreaks have been caused by bacterial toxins in 2021 (Figure 1). Among toxins from defined species, the highest outbreaks number were linked to *Bacillus cereus* (n=87), while *Clostridium perfringens* and *Staphylococcus aureus* toxins reported the highest number of cases-deaths (n=778;4) and hospitalisations (n=51) respectively (European Food Safety Authority & European Centre for Disease Prevention and Control, 2022).

Type of agent		Outbreaks					Cases of illness						
		Total (Strong-evidence)	% of total[a]	Reporting rate per 100,000			Human cases		Mean outbreak size (cases) and range (min–max)	Hospitalisations		Deaths	
		N		2021[b]	2020	2017–2019[c] (Mean)	N	% of total[a]		N	% of cases[d]	N	% of cases[d]
Bacteria	*Aeromonas*	1 (1)	0.02	< 0.01	0	< 0.01	19	0.10	19.0 (–)	0	0	0	0
	Brucella	1 (0)	0.02	< 0.01	< 0.01	< 0.01	2	0.01	2.0 (–)	2	100	0	0
	Campylobacter[e]	249 (20)	6.2	0.06	0.07	0.10	1,051	3.2	4.2 (2–39)	134	12.7	6	0.57
	Cronobacter sakazakii	1 (1)	0.02	< 0.01	0	< 0.01	4	0.01	4.0 (–)	4	100	1	25.0
	Escherichia coli other than STEC	27 (4)	0.70	0.01	< 0.01	< 0.01	327	1.0	12.1 (2–85)	44	13.5	0	0
	Listeria monocytogenes	23 (8)	0.60	0.01	< 0.01	< 0.01	104	0.30	4.5 (2–11)	48	46.2	12	11.5
	Salmonella	773 (143)	19.3	0.17	0.16	0.27	6,755	20.8	8.7 (2–728)	1,123	16.6	1	0.01
	Shiga toxin-producing *E. coli* (STEC)	31 (5)	0.80	0.01	0.01	0.01	275	0.80	8.9 (2–76)	47	17.1	0	0
	Shigella	11 (1)	0.30	< 0.01	< 0.01	0.01	63	0.20	5.7 (2–21)	4	6.3	0	0
	Vibrio cholera (non-toxigenic)	1 (1)	0.02	< 0.01		< 0.01	47	0.10	47.0 (–)	1	2.1	0	0
	Vibrio parahaemolyticus	3 (1)	0.10	< 0.01	< 0.01	< 0.01	10	0.03	3.3 (2–6)	0	0	0	0
	Yersinia	21 (4)	0.50	< 0.01	< 0.01	< 0.01	125	0.40	6.0 (2–26)	14	11.2	0	0
	Other bacteria/ unspecified	1 (0)	0.02	< 0.01	< 0.01	< 0.01	16	0.05	16.0 (–)	0	0	0	0
	Subtotal[e]	**1,143 (189)**	**28.5**	**0.25**	**0.24**	**0.39**	**8,798**	**27.0**	**7.7 (2–728)**	**1,421**	**16.2**	**20**	**0.23**
Bacterial toxins	*Bacillus cereus* toxins	87 (15)	2.2	0.02	0.02	0.04	679	2.1	7.8 (2–93)	9	1.3	1	0.15
	Clostridium botulinum toxins	7 (4)	0.20	< 0.01	< 0.01	< 0.01	24	0.10	3.4 (2–8)	15	62.5	0	0
	Clostridium perfringens toxins	40 (20)	1.0	0.01	0.01	0.02	778	2.4	19.5 (2–69)	25	3.2	4	0.51
	Staphylococcus aureus toxins	61 (20)	1.5	0.01	0.01	0.04	640	2.0	10.5 (2–62)	51	8.0	0	0
	Bacterial toxins, unspecified	484 (13)	12.1	0.11	0.08	0.13	4,257	13.1	8.8 (2–329)	210	4.9	2	0.05
	Subtotal	**679 (72)**	**17.0**	**0.15**	**0.12**	**0.18**	**6,378**	**19.6**	**9.4 (2–329)**	**310**	**4.9**	**7**	**0.11**

(a): Percentage out of the total number of cases reported in the EU.
(b): Data on foodborne outbreaks from the United Kingdom (Northern Ireland) are taken into account for 2021. In accordance with the agreement on the withdrawal of the United Kingdom from the EU, and in particular with the Protocol on Ireland/Northern Ireland, the EU requirements on data sampling are also applicable to Northern Ireland.
(c): Data on foodborne outbreaks from the United Kingdom are taken into account for 2017–2019, because the United Kingdom was an EU Member State, but it became a third country on 1 February 2020. (d): Percentage out of the total number of cases caused by the causative agent.
(e): For one outbreak, information on cases was not available. This outbreak was excluded from the calculation of the mean outbreak size.

Figure 1: 2021 EU key statistics on foodborne outbreaks, human cases, hospitalizations and deaths, by causative agents. Outbreaks are classified as 'strong evidence' or 'weak evidence' according to the strength of evidence linking a putative food vehicle to the disease (European Food Safety Authority & European Centre for Disease Prevention and Control, 2022).

The EFSA-ECDC joint report provided also details on food vehicles most frequently implicated in foodborne outbreaks, thereby highlighting on which sources control policies should focus more to mitigate outbreaks public health impact, either at primary production level or across food preparation sectors (Figure 2). In 2021, the most frequently reported food category in foodborne outbreaks belonged to 'mixed foods', encompassing a wide range of food products, such as bakery, buffet meals, sweet and chocolate, and others, which reported the highest number of cases and hospitalizations with *Salmonella*, *Campylobacter*, and ETEC among the major causative agents. Nevertheless, due to the wide range of ingredients included in this category, the primary source of contamination is challenging to identify. Following 'mixed foods', the second most reported category were 'meat and meat products'. More in deep, 'Pig meat and products thereof' ranked first among this group, being mostly vehicle of Salmonella (n= 14 outbreaks) and *Clostridium perfringens* toxins (n=3 outbreaks). After 'meat and meat products', other major concerning 'Fish and fishery products', 'Foods of non-animal origin' and 'Eggs and egg products'.

Interestingly, among 'Foods of non-animal origin' the implication of 'vegetables and juices and other products thereof' in foodborne outbreaks significantly increased compared to 2020 and the pre-pandemic years, with *Salmonella* identified as a major foodborne infection. Furthermore, 12 additional outbreaks were reported for 'Milk and milk products' group in 2021 compared to the previous year as a result of the increasing number of outbreaks from cheese (n=14 more than 2020), mainly linked to *Salmonella* and *S. aureus* toxins (European Food Safety Authority & European Centre for Disease Prevention and Control, 2022).

To summarize, trends in the prevalence are heavily impacted not only by the extent of food contamination at the consumer level, but also by changes in human behaviors and data collection. (Adinolfi et al., 2016).

Type of vehicle	Strong-evidence outbreaks							
	Outbreaks		Cases		Hospitalisations		Deaths	
	N	% of total	N	% of total	N	% of total	N	% of total
Composite foods, multi-ingredients foods and other foods								
Mixed foods	76	21.4	2,156	30.8	181	24.4	2	15.4
Bakery products	18	5.1	258	3.7	52	7.0	0	0
Buffet meals	5	1.4	205	2.9	33	4.5	0	0
Sweets and chocolate	2	0.60	34	0.50	8	1.1	0	0
Other foods	5	1.4	103	1.5	2	0.30	0	0
Subtotal	**106**	**29.9**	**2,756**	**39.3**	**276**	**37.2**	**2**	**15.4**
Meat and meat products								
Pig meat and products thereof	22	6.2	347	5.0	69	9.3	3	23.1
Broiler meat (*Gallus gallus*) and products thereof	21	5.9	202	2.9	42	5.7	0	0
Meat and meat products, unspecified	17	4.8	237	3.4	18	2.4	2	15.4
Bovine meat and products thereof	13	3.7	201	2.9	12	1.6	0	0
Other or mixed red meat and products thereof	2	0.60	11	0.20	0	0	0	0
Other, mixed or unspecified poultry meat and products thereof	2	0.60	14	0.20	0	0	0	0
Subtotal	**77**	**21.7**	**1,012**	**14.4**	**141**	**19**	**5**	**38.5**
Fish and fishery products								
Fish and fish products	30	8.5	190	2.7	41	5.5	4	30.8
Crustaceans, shellfish, molluscs and products thereof	25	7.0	171	2.4	13	1.8	0	0
Subtotal	**55**	**15.5**	**361**	**5.2**	**54**	**7.3**	**4**	**30.8**
Food of non-animal origin								
Vegetables and juices and products thereof	34	9.6	1,700	24.3	131	17.7	0	0
Cereal products including rice and seeds/pulses	9	2.5	194	2.8	17	2.3	0	0
Fruit, berries and juices and products thereof	2	0.60	15	0.20	0	0	0	0
Subtotal	**45**	**12.7**	**1,909**	**27.3**	**148**	**20.0**	**0**	**0**
Eggs and egg products	42	11.8	439	6.3	90	12.1	1	7.7
Milk and milk products								
Cheese	18	5.1	235	3.4	11	1.5	0	0
Dairy products (other than cheeses)	6	1.7	119	1.7	7	0.90	1	7.7
Milk	4	1.1	55	0.80	13	1.8	0	0
Subtotal	**28**	**7.9**	**409**	**5.8**	**31**	**4.2**	**1**	**7.7**

Figure 2: Frequency distribution of strong-evidence foodborne outbreaks according to food vehicle, in 2021 (European Food Safety Authority & European Centre for Disease Prevention and Control, 2022).

In recent years, an increasing attention has been given to the way food is produced and consumed, while respecting health protection, food quality and trade dynamics.

Food policy within the EU framework has been handled by each MS until the late 1990s. Following a series of geographically spread food crisis (e.g., the Bovine Spongiform Encephalitis, *E. coli*, etc.) these competences were transferred from the MS to European Institutions. Consequently, general principles and requirements regarding food and feed law were implemented with specific

Regulations, Directives and decisions. The European Commission set up a comprehensive and integrated regulation of individual production phases as well as operators' behaviour, covering all sectors of the food chain from primary production to food processing, storage and retail.

As consumers are increasingly focused in buying food products capable of reducing heath damage, this system of regulations drew the attention at assuring healthy and safe food to the population, in the respect of both food producers and consumers interests. Efforts are therefore directed to food safety and to establish advanced procedures to reduce risks derived from food contamination. According to this approach, a food product could be considered as "safe" if it does not present any risk, or present risks reduces and acceptable. Moreover, every product placed on the market and intended for consumption needs to fulfil specific safety requirements (Pettoello-Mantovani & Olivieri, 2022).

In 2002, Regulation No 178/2002 was adopted by the European Parliament and the Council, which sets out a meaningful and coherent framework concerning general principles and requirements of food law both at Union and national levels. With this regulation, the free movement of food and food animals is strongly underpinned by food safety principles', protecting animal health and welfare and adopting a systematic control of food and feed movement along the entire food chain (from farm to table) (European Commission, 2020b).

Another milestone in food safety regulations was set up with the establishment of the European Food Safety Authority (EFSA), whose roles are shaped within article 23 of Regulation No. 178 of 2002. Basically, EFSA is an independent agency providing support and scientific advice to protect European consumers from food-related risks. EFSA activities are therefore concerned with the collection and integration of scientific evidence regarding existing and emerging risks in the area of food and feed safety, animal health and welfare as well as plant health (European Union, 2020).

As far as food safety is concerned, also MS are included among European legislation, being responsible of organizing its own system according to the European coordinated system of food safety. The connection between the European system and those of the individual MS and between the latter and any local legislation suggest a nested cooperation among the current European food safety discipline (Pettoello-Mantovani & Olivieri, 2022).

Moreover, the EU has set up the Rapid Exchange of Information System (RAPEX), a rapid alert system for unsafe consumer products and consumer protection. This system was developed to facilitate coordination between businesses and the competent authorities of MS (European Commission, 2020a). Additionally, the Rapid Alert System of the European Union (RASFF) was to provide a real time notification of direct or indirect risks to health deriving from the consumption of food or feed. The European Commission, the EFSA and the MS are together networked of this alert system, aimed at withdrawal products considered dangerous to human or animal health (Food Standards Scotland, 2020).

At the global scale, the World Health Organization (WHO) launched the International Food Safety Authorities Network (INFOSAN) in 2004, in cooperation with the Food and Agriculture Organization (FAO) of the United Nations. INFOSAN represents a global tool to improve food safety and mitigate the burden of foodborne disease by facilitating urgent international communication during food safety

emergencies. In addition, since the International Health Regulations (IHR [2005]) came into force in 2007, INFOSAN has been recognized as a crucial tool to assist countries in developing the core capacities required for food safety emergency preparedness and response (Savelli et al., 2019).

1.2. Regulation EU 2073/2005 for microbiological criteria

Food safety control includes producers, manufacturing facilities, and food service businesses associated to a particular food item sold within the MS both if produced at national level or imported. To date, international trade of food items has extremely increased, suggesting that a significant proportion of manufacturers and consumers living in importing nations may receive raw materials or finished products from manufacturing facilities located in other countries. Increasing international trade, modifying dietary habits together with food crises which took place over the last 20 years (Bovine Spongiform Encephalopathy, dioxins, foot and mouth disease, etc.) boosted consumers sensitivity on food safety issues and risk managers to strengthen and harmonize food safety control procedures (Manfreda & De Cesare, 2014). In addition to the risk analysis framework laid out by Regulation (EC) 178/2002, the Commission Regulation (EC) 2073/2005 set out microbiological criteria used to assess the acceptability of food. A microbiological criterion is defined as a guideline that determines the acceptability of a product, a batch of food or a process based on the quantity of toxins/metabolites produced by microorganisms per unit(s) of mass, volume, area, or batch, and/or the absence, presence, or number of such organisms. Additionally, microbiological criteria include sampling plans, parameters of acceptability, analytical method to be used and point of the food chain to be sampled. In particular:

- sampling plan that specifies the size of the analytical unit (e.g., 25 g) and the number of field samples that shall be taken (i.e., n);
- microbiological thresholds (m and M or absence) thought to be suitable for a specific hazard/food combination at the designated site through the food chain;
- the number of analytical units (c) that must comply with these limits as well as the appropriate measures in case the requirement is not satisfied.
- Analytical method. The usage of alternative methods is allowed on the assumption that they give equal guarantees in terms of food safety. Furthermore, they must be certified in accordance with globally established protocols (e.g., EN/ISO standard 16140), and their usage must be authorized by the appropriate authorities.

Regulation (EC) 2073/2005 displays two types of microbiological criteria, such as food safety criteria and process hygiene criteria. Food safety criteria should be applied to determine whether a product or batch of food is safe. These requirements must be met for the duration of the product's shelf life, and if they are not, the food company operator will be forced to take the product off the market. By contrast, process hygiene criteria are used to assess if the production processes are working hygienically by establishing indicative contamination thresholds above which corrective measures are needed to keep the process's hygiene compliant with food law. Whether a process hygiene criterion is not fulfilled, the product may still be distributed, but the food company owner must assess

the production procedures and boost process hygiene to guarantee that future production will comply with the standard (Food Standards Agency, 2022).

Microbiological criteria are displayed by Annex I of the Regulation, which is divided into three chapters, such as (i) food safety criteria, (ii) process hygiene criteria (ii) and (iii) rules for sampling and preparation of test samples.

Chapter 1 and 2 outline specific sampling strategies and remedial measures for the microbiological criteria. Food safety criteria are referred to the following microorganism:

- *Salmonella* (meat products, poultry, dairy products, eggs, fish live bivalve, ready to eat (RTE) raw fruits and juices, special purpose foods);
- *Salmonella enterica* serovars Enteritidis and Typhimurium (fresh poultry meat);
- *Listeria monocytogenes* (RTE foods with different criteria depending on the food characteristics: supporting/not supporting growth; and also depending on the destination: intended/not intended for infants and for special medical purposes);
- *Staphylococci enterotoxins* (cheeses, milk and powder whey);
- *Enterobacter sakazakii* (Infant formula and food);
- *E. coli* (Live Bivalve).

Process hygiene criteria are established for different category of products, such as (i) meat and products thereof, (ii) milk and dairy products, (iii) egg products, (iv) fishery products, (v) vegetables and (vi) fruits and products thereof. Like those concerning food safety, each food category within process hygiene criteria is referred to specific microorganisms:

- Aerobic colony count and *Enterobacteriaceae* (Meat Carcasses);
- *Salmonella* spp. (Meat Carcasses);
- Aerobic colony count and *E. coli* (minced meat, mechanically separated meat, meat products);
- *Enterobacteriaceae* (milk and milk products, ice-cream, foods for special medical purposes, egg-products);
- *E. coli* (dairy products, fish products, fruit and vegetables, non-pasteurised juices);
- Coagulase positive *Staphylococcus* (cheese and cooked fish products);
- *Campylobacter* (poultry carcasses).

Finally, Chapter 3 outlines specific rules provided for bacteriological sampling in slaughterhouses and at premises producing minced meat and meat preparations. According to ISO reference methods and the guidelines of the Codex Alimentarius, sampling rules are set out for carcasses of cattle, pigs, sheep, goats, horses, and poultry along with sampling frequencies for carcasses, minced meat, meat preparations and mechanically separated meat.

All food industry operators, including retailers and caterers, who process, manufacture, handle, or distribute food are subject to this Regulation, thus being responsible of checking that foodstuffs meet the pertinent microbiological criteria and taking the necessary steps if a product is found to fall short of any of them (Food Standards Agency, 2022). Moreover, the competent authority shall ensure compliance with the rules and criteria imposed by the Regulation, without limiting its rights to perform additional sampling and analyses for the purpose of detecting and estimating other

microorganisms, their toxins, or metabolites, either as a verification of processes, for food suspected of being unsafe, or in the context of a risk analysis. Regular reviews of the microbiological criteria must be corroborated by advancements in science and technology, emerging pathogenic microorganisms in foodstuffs as well as results from risk assessments.

1.3. Principles of risk assessment and traceability

According to Regulation (EC) 178/2002, a risk is identified by evaluating the probability and severity of the adverse health effect of the food or feed on health, deriving from the presence of a hazard. A hazard is therefore defined as the chemical, physical or biological agent contained in a food or feed or condition in which a food or feed is found capable of causing an adverse health effect. The Regulation also state that it is strictly forbidden to place on the market any food considered as injurious to health or unfit for human consumption (Pettoello-Mantovani & Olivieri, 2022).

According to food safety requirements, by which methods the EU protects consumer health from food hazards? The general principle for consumer protection is included in the process of risk analysis.

The risk analysis encompasses three interconnected components: risk assessment, risk management and risk communication.

The risk assessment is carried out by a scientifically based procedure, aimed at assessing the exposure to the hazard and the risk as well as the probability and the severity of the harmful health effect. This process must be undertaken in an independent, objective and transparent manner based on the best available science. The European Agency in charge of the risk assessment is EFSA, which collect and gather communications from MS or national authorities, consumers, food businesses, the academic community and those interested in food safety. The risk management musts consider the results of risk assessment, to evaluate policy alternatives in consultation with interested parties (e.g., the European Commission, Member State authorities) and through the analysis between the alternatives of intervention and the adoption of restrictive measures and appropriate preventive and control choices to protect health. Finally, the last important step is the risk communication, consisting in the interactive exchange of information and opinions as regards hazards and risks between managers, consumers, food companies and other interested parties. This communication includes the explanation of risk assessment findings and the basis of risk management decisions. (Chatzopoulou et al., 2020; Pettoello-Mantovani & Olivieri, 2022)

Another obligation related to food safety is that producers must guarantee the traceability of food products from their origin to the consumer's table. The traceability obligation was firstly introduced within Regulation No. 178 of 2002 in response to the emergency in Europe of Creutzfeldt-Jakob disease among the beef sector, commonly referred to as "mad cow disease", and concerns the flow of raw materials and components among the production process of an individual food business. If potentially dangerous situations for the consumer take place during the production chain, it is essential to identify the product placed on the market as fast as possible and withdraw it even in case of exportation to other countries. In particular, the traceability system warrants the identification of the person responsible for the danger produced and the damage caused. Consequently, this facilitates the

identification of who is obliged to comply with the regulatory provisions for the protection of the safety of the food product, followed by the obligation to communicate any dangerous situation to consumers or to those responsible for withdrawing it from the market. As regard to the food imported from third countries, whether the risk could not be properly handled with measures adopted by MS, the traceability system allows to adopt appropriate emergency measures at Union level for food and feed imported from a third country (Pettoello-Mantovani & Olivieri, 2022). For this purpose, the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) entered into force on 1 January 1995 as regulatory measures of health protection against risks contained in food and agricultural products. The basic aim of the SPS Agreement is to maintain the right of any government to provide the level of health protection it deems appropriate, but to ensure that these rights are not misused for protectionist purposes and do not result in unnecessary barriers to international trade. These measures can take many forms, such as requiring products to come from a disease-free area, inspection of products, specific treatment or processing of products, setting of allowable maximum levels of pesticide residues or permitted use of only certain additives in food (Prévost & Van den Bossche, 2005).

Overall, the introduction of the traceability among the European legislation has led to an increased engagement to supervision, risk assessment and review of the substances used and enhanced the control of food at all stages of the food production process, including production, processing, transport and distribution (Fusco et al., 2020).

2. Pursuing food safety in artisanal dairy and meat fermented productions

2.1. Moving towards tradition and typicality of artisanal food products

New production criteria have been required since the 1980s to undergo the increasing volumes of foods processed with high hygienic standards. Consequently, this trend has frequently led to drastic changes to food processing, thereby changing the relationship between the production environment and product characteristics. For instance, a shift from artisanal or small-scale productions to those with a high degree of automation has been addressed, having an overall impact on the entire agri-food chain (Wilkinson, 2004).

Nevertheless, in recent years artisanal food products have become increasingly popular and represent by far an intrinsic element of European culture, identity, and heritage. This boost in popularity derives from their rising support for social and environmental issues, strictly connected to sustainability, traceability, and a loyalty to everything local (Cirne et al., 2019).

A definition of "traditional" foods was established by the European Commission in 2006: "*Traditional means proven usage in the community market for a time period showing transmission between generations*; *this time period should be the one generally ascribed as one human generation*, *at least* 25 *years*" (EU, 2006).

Moreover, the Italian Ministry of Agriculture delineated a formal definition for "traditional food products", intended as "*Agrifood products whose methods of processing, storage and ripening are*

consolidated with time according to uniform and constant local use". Whilst both definitions attempt to shape the principal traits of traditional food items, they lack the consumer's perspective.

From the consumers' standpoint, the three major leading forces in artisanal food success are flavour, health, and ethical responsibility. When seeking for a food product, taste has always been and will continue to be one of the most important selection criteria. Flavour and taste of artisanal food are generally perceived better than industrial alternatives, thus raising consumer's acceptability. Health issues are also becoming more prevalent across the food industry, with a wide range of food companies more prompt to decrease fat, cut out sugar, and lower caloric intake. Artisanal foods frequently meet this health demand, also by enhancing their product with certifications and nutritional claims. Ethical responsibilities generally may encompass responsible farming and sourcing, job security, close and direct relationships, donating and supporting to non-profits, community support, environmental consciousness and sustainability (Cirne et al., 2019).

Moreover, another factor that may be appealing to consumers is the product location of origin, or the one of its raw materials, as well as their source, since in most cases typical local animal breeds and old varieties of plants are employed (Hajdukiewicz, 2014; Lebert et al., 2007).

However, traditional foods are in general more expensive than industrial ones because of the higher cost in production methods and raw ingredients. The EU acknowledged the importance of supporting local food producers and allowing them to safeguard their goods and know-how by setting up Regulation No 1151/2012 on quality schemes for agricultural products and foodstuffs (Trichopoulou et al., 2007). This regulation allows unique food products to be protected with their registration as Protected Designations of Origin (PDO), Protected Geographical Indications (PGI) and Traditional Specialties Guaranteed (TSG). These registrations give the opportunity to enjoy a 'recognition of quality' status, by promoting and protecting name and traditional processes of quality agricultural products and foodstuffs (Kizos et al., 2017).

Following the increasing interest in traditional foods in Europe, PDO, PGI and TSG product lists are expanding (Lücke & Vogeley, 2012).

Nevertheless, from an industry perspective there is a lack of regulations/guidelines required to certify differences occurred in pre-production, production, and post-production. Because of this, it could be challenging for artisanal producers to claim and recognize artisanal products quality and differentiate them from the industrial ones (Cirne et al., 2019). Moreover, the absence of specific regulations is even worsened in the case of small artisanal facilities, thus resulting in non-standardized productions, with marked variations between each one, as well as also addressing several safety issues.

2.2. Food fermentations overview

Fermentation has been a valuable means of food preservation and functional/nutritional improvement since ages (Marco et al., 2017). In the food microbiology field, the term fermentation is broadly used to describe the employment of microbes or their related enzymes to convert raw materials into finished products (Settanni & Moschetti, 2014). Basically, to gain energy and support their anabolic activities, fermenting bacteria decrease the content of carbohydrates and other macromolecules

accessible in the substrate by accumulating catabolic metabolites (e.g., lactic acid, ethanol). A wide range of effects and modifications are generated by fermentation processes. First, biological processes together with the potential release of antimicrobial compounds could minimize the burden of undesirable microbial developments, suggesting an enhancement in product shelf-life and safety level (Russo et al., 2017a). Second, palatability and sensory attributes of the fermented matrices are strongly influenced by primary and secondary metabolites (Romano et al., 2015). Finally, the nutritional content of the food products is significantly altered by microbes, owing to the creation of biomolecules of nutritional importance synthetized by fermenting cells. In this regard, the fermentation process improves protein and carbohydrate digestibility as well as vitamin and mineral bioavailability (Capozzi et al., 2012a; El Sheikha & Hu, 2020).

Thanks to the creation of new desirable organoleptic qualities, fermented food products are more digestible and attractive than previous substrates.

Notably, a complex microbial ecosystem is associated with the huge diversity in terms of raw materials, fermentative behaviour and obtained products (Tamang et al., 2016). These are primarily characterised by hundreds of different species of bacteria, particularly lactic acid bacteria (LAB), yeasts, and filamentous fungi, whose fermentation confers quality features such as palatability, high sensory quality, structure and texture, stability, nutritional and healthful qualities, and, eventually, potential probiotic properties to the resulting products (Settanni & Moschetti, 2014).

Figure 3 depicts examples of prokaryotic and eukaryotic genera/species involved in fermentative processes across nine food/beverages fermented categories, proposed by Tamang et al. (2016). This list emphasizes the vast micro-biodiversity associated with the various edible matrices subjected to the fermentative process.

Furthermore, given that each species has substantial intraspecific diversity and that many desired and undesirable microbial traits connected with food fermentations are strain-dependent, the potentially significant influence of such diversity on global food quality becomes evident.

To summarize, the microbial diversity associated with the fermenting matrix has the potential to alter the key quality and safety attributes of a food products (Capozzi et al., 2017). This may encompass: (i) food safety, by inflecting the content of biological and chemical contaminants, (ii) sensorial quality, by producing volatile organic compounds and prompting the taste and the texture and (iii) nutritional quality, by modulating macro- and micro-nutrients thus affecting their digestibility and bioavailability (Taboada et al., 2017).

Fermented foods and beverages are a worldwide sector with rising importance in human nutrition and the economy due to their vast geographical distribution and relevance in terms of consumption across populations. A wide diversity of fermented foods are globally diffused or linked to a national/continental origin, whereas a significant variety in terms of raw materials and fermentation types has a regional dispersion (Tamang et al., 2016).

Alcoholic drinks, bread and baked items, cheeses and fermented milks, table olives and other processed vegetables as well as fermented meat and fish products are among the most popular fermented foods (Wood, 1997).

Major Groups of Globally Fermented Foods	Microorganisms Involved in the Fermentation Process
Fermented cereals	*Lactococcus* sp., *Leuc. mesenteroides*, *Lb. delbrueckii*, *Lb. fermenti*, *Lb. coryniformis*, *Leuconostoc* sp., *Ped. acidilactis*, *Ped. cerevisae*, *Streptococcus* sp., *Ent. faecalis*, *Ent. cloacae*, *Weissela* sp., *Bacillus amyloliquefaciens*, *Klebsiella pneumoniae*, *Aerobacter* sp., *Candida cacaoi*, *Cand. fragicola*, *Cand. glabrata*, *Cand. kefyr*, *Cand. pseudotropicalis*, *Cand. sake*, *Cand. tropicalis*, *Debaryomyces hansenii*, *Deb. tamarii*, *Issatchenkia terricola*, *Kluyveromyces marxianus*, *Sacch. cerevisiae*, *Torulopsis candida*, *Tor. holmii*, *Monascus purpureus*, *Rhizopus* sp., *Cephalosporium* sp., *Mucor* sp., *Fusarium* sp., *Penicillum* sp., *Aspergillus* sp., *Endomycopsis* sp., *Hansenula* sp.
Fermented vegetables and bamboo shoots	*Leuc. mesenteroides*, *Leuc. citreum*, *Leuc. gasicomitatum*, *Leuc. fallax*, *Leuc. kimchii*, *Leuc. inhae*, *W. koreensis*, *W. kimchii*, *W. cibaria*, *Lb. plantarum*, *Lb. sakei*, *Lb. delbrueckii*, *Lb. buchneri*, *Lb. brevis*, *Lb. fermentum*, *Ped. acidilactici*, *Ped. pentosaceus*, *Lc. lactis*, *Ent. durans*, *Tetragenococcus halophilus*, *Bacillus subtilis*, *B. lichniformis*, *B. coagulans*, *B. cereus*, *B. circulans*, *B. firmus*, *B. pumilus*, *B. sphaericus Candida* sp., *Halococcus* sp., *Haloterrigena* sp., *Kluyveromyces* sp., *Lodderomyces* sp., *Natrialba* sp., *Natronococcus* sp., *Pichia* sp., *Saccharomyces* sp., *Sporisorium* sp., *Trichosporon* sp., *Pseudomonas* sp., *Halorubrum orientalis*, *Halosarcina pallid*, *Sphingobium* sp., *Thalassomonas agarivorans*
Fermented legumes	*Bacillus subtilis*, *B. brevis*, *B. circulans*, *B. coagulans*, *B. licheniformis*, *B. pumilus*, *B. sphaericus*, *Lysinibacillus fusiformis Rhiz. oligisporus*, *Rhiz. arrhizus*, *Rhiz. oryzae*, *Rhiz. stolonifer*, *Asp. niger*, *Citrobacter freundii*, *Enterobacter cloacae*, *K. pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, *Pseudomas fluorescens*, *Lb. fermentum*, *Lb. lactis*, *Lb. plantarum*, *Lb. reuteri*, *Pantoea agglomerans*, *P. gaananatis*, *Enterococcus* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Asp. oryzae*, *Asp. flavus*, *Asp. fumigatus*, *Asp. niger*, *Asp. retricus*, *Asp. spinosa*, *Asp. terreus*, *Asp. wentii*, *Botrytis cineara*, *Ped. halophilus*, *Staphylococcus* sp.
Fermented roots/tubers	*Bacillus* sp., *Lb. plantarum*, *Leuc. mesenteroides*, *Lb. cellobiosus*, *Lb. brevis; Lb. coprophilus*, *Lc. lactis; Leuc. lactis*, *Lb. bulgaricus*, *Klebsiella* sp., *Leuconostoc* sp., *Corynebacterium* sp., *Candida* sp., *Micrococcus* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Moraxella* sp., *Rhizopus* sp.
Fermented milk products	*Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Lb. alimentarius*, *Lb. biofermentans*, *Lb. brevis*, *Lb. delbrueckii* subsp. *delbrueckii*, *Lb. delbrueckii subsp. lactis*, *Lb. farciminis*, *Lb. helveticus*, *Lb. casei*, *Lb. plantarum*, *Lb. salivarius*, *Leuconostoc* spp., *Strep. thermophilus*, *Ent. durans*, *Ent.faecalis*, *Ent. faecium*, *Ped. pentosaceous*, *Ped. acidilactici*, *Bifidobacterium* spp., *Staphylococcus* spp., *Brevibacterium linens*, *Propionibacterium freudenreichii*, *Weissella confusa*, *Candida* sp., *Saccharomycopsis* sp., *Debaryomyces hansenii*, *Geotrichum candidum*, *Penicillium camemberti*, *P. roqueforti*, *Pichia kudriavzevii*
Fermented and preserved meat products	*Lb. pentosus*, *Lb. plantarum*, *Lb. brevis*, *Lb. paracasei*, *Lb. fermentum*, *Lb. acidipiscis*, *Lb. farciminis*, *Lb. rossiae*, *Lb. fuchuensis*, *Lb. namurensis*, *Lc. lactis*, *Lb. sakei*, *Leuc. citreum*, *Leuc. fallax*, *Ped. acidilactici*, *Ped. pentosaceus*, *Ped. stilesii*, *W. cibaria*, *W. paramesenteroides*, *Ent. faecalis*, *Ent. faecium*, *Ent. hirae*, *Bacillus subtilis*, *B. mycoides*, *B. thuringiensis*, *Staphylococcus spp.*, *Micrococcus* sp.
Fermented, dried, and smoked fish products	*Lc. lactis*, *Lb. plantarum*, *Lb. pobuzihii*, *Lb. fructosus*, *Lb. amylophilus*, *Lb. coryniformis*, *Ent. faecium*, *Ent. faecalis*, *Bacillus subtilis*, *B. pumilus*, *B indicus*, *Micrococcus* sp., *Staphylococcus cohnii* subsp. *cohnii*, *S. carnosus*, *Strep. faecalis*, *Sarcina* sp., *Corynebacterium* sp., *Tetragenococcus halophilus* subsp. *flandriensis*, *Pseudomonas* sp., *Halococcus* sp., *Halobacterium salinarium*, *H. cutirubrum*, *Clostridium irregular*, *Azorhizobium caulinodans*, *Candida* sp., *Saccharomycopsis* sp.
Miscellaneous fermented products	*Acetobacter aceti* subsp. *aceti*, *Acetobacter pasteurianus*, *Acetobacter polyxygenes*, *Acetobacter xylinum*, *Acetobacter malorum*, *Acetobacter pomorum*, *Candida lactis-condensi*, *Candida stellata*, *Hanseniaspora valbyensis*, *Hanseniaspora osmophila*, *Saccharomycodes ludwigii*, *Sacch. cerevisiae*, *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus*, *Zygosaccharomyces lentus*, *Zygosaccharomyces mellis*, *Zygosaccharomyces Pseudorouxii*, *Zygosaccharomyces rouxii*
Alcoholic beverages	*Saccharomyces cerevisiae*, *Candida colliculosa*, *C. stellata*, *Hanseniaspora uvarum*, *Kloeckera apiculata*, *Kl. thermotolerans*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia fermentans*, *Schizosaccharomyces pombe*, *Hanseniaspora uvarum*, *Oenococcus oeni*, *Lb. plantatum*

Ent., Enterococcus; *Lb., Lactobacillus*; *Lc., Lactococcus*; *Leuc., Leuconostoc*; *W., Weissella*; *Ped., Pediococcus.*

Figure 3: Example of microbial dimension involved in fermentative processes of the main categories of globally fermented foods and beverages (Greppi et al., 2013; Petruzzi et al., 2017; Tamang et al., 2016).

2.2.1. Return to artisanal fermentations

In the assumption that food reflects people's origin, culture and traditions, societies that can preserve their traditional foods have thus maintained their history and cultural heritage.

Traditional fermented foods derive from biotechnological processes that take advantage of the natural microflora associated with typical raw ingredients from plants or animals, or by introducing selected starting cultures. Moreover, they represent an important element of the human diet across the world, notably in Latin America, Asia, and Africa (El Sheikha & Hu, 2020).

The widespread popularity of traditional fermented foods is strongly connected to indigenous fermentation techniques, a reflection of the link with a specific territory marked by distinctive technological features.

In general, fermented foods produced at industrial levels, whether in different locations, have unchanging organoleptic characteristics. As a result, microorganisms must have repeatable and reproducible technological performances to achieve product uniformity. Local fermented foods, on the other hand, are created at the artisanal level using autochthonous bacteria that are tailored to the production location (environment), local raw materials (substrates), and the customary methodology (technology). Apparently, these microorganisms may not be able to replicate their performances under diverse environments (Settanni & Moschetti, 2014).

By virtue of their increased organoleptic features, traditional fermented foods give pleasure and diversity to monotonous diets, thus boosting consumer's acceptability (El Sheikha & Hu, 2020).

Assumed the deep influence of microorganisms in fermentation processes, particularly in traditional fermented productions the microbial consortia are of paramount importance.

Indeed, indigenous microorganisms that are intended as autochthonous are frequently connected with a certain place/environment, thus acting as a typical ingredient in fermented foods (Dubos et al., 1965). These bacteria are frequently responsible for the production of characteristic flavor and are part of the heritage linked with a particular food product (Piraino et al., 2005; Schuller et al., 2005).

Because the microbiota of traditional fermented foods affects their final quality, they assist as the producer's "coworkers" and are part of the production traditions that have been formed over time, thus providing a direct relationship between food and the historical and social conditions unique to a particular place (Settanni & Moschetti, 2014).

When conceiving the idea of typicality and identity, the incorporation of local raw materials, including autochthonous microorganisms, plays a crucial role in consumer perception of quality.

Nevertheless, raw ingredients and microorganisms employed in traditional fermented productions not only contribute to the unique flavours, but also to food safety.

Whilst microbial populations that produce organic acids, carbon dioxide, ethanol, hydrogen peroxide and diacetyl, antifungal compounds, bacteriocins, and antibiotics during the fermentation process contribute to food's protection, in some circumstances it does not ensure the absence of unwanted microorganisms in the final mixture (Settanni & Corsetti, 2008). Indeed, like fresh or alternatively processed foods, fermented foods may be exposed to many hazards to human health. Among them, it is essential to distinguish biological risks from chemical risks of microbiological origin. The formers are based on biological contaminants, such as microbial foodborne pathogens linked to principal categories of fermented foods. Microbial pathogens have been linked to a variety of fermented foods, including cheese, sausages, fermented seafood, and fermented cereals, suggesting fermented products based on animal matrices mostly critical to contamination (Adekoya et al., 2017; Nout, 1994; Sivamaruthi et al., 2019).

Pathogenic microbes may generally include *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Aeromonas* spp., *Klebsiella* spp., *Campylobacter* spp., *Shigella* spp., *Bacillus cereus*, *Proteus mirabilis*, *Staphylococcus aureus*, *Proteus penneri*, *Enterococcus faecalis*, and *Staphylococcus saprophyticus* (Kumar et al., 2019; Sivamaruthi et al., 2019; Walsh et al., 2017).

For instance, foodborne pathogens such as *Listeria monocytogenes* and enterohaemorrhagic *Escherichia coli* have been found to easily adapt to acidic and salty environments typically used in food fermentations (Mataragas et al., 2015; McLeod et al., 2016).

On the other hand, further hazards may include toxic by-products of microbial origin. These chemical contaminants comprises a variety of microbial-related molecules including mycotoxins (e.g., aflatoxins, deoxynivalenol, fumonisins, nivalenol, ochratoxin, zearalenone), biogenic amines (cadaverine, histamine, isoamylamine, phenylethylamine, putrescine, and tyramine), ethyl carbamate, and cyanogenic glycosides (Nout, 1994; Sivamaruthi et al., 2019). However, even some LAB strains may release biogenic amines, which can induce headaches, nausea, and even anaphylactic shock in sensitive individuals (McCabe-Sellers et al., 2006).

Besides the mentioned pathogens and chemical hazards of microbial origin, an additional risk which potentially impairs the safety of food fermentations is the occurrence and/or transfer of virulence and antimicrobial resistance (AMR) traits harboured by bacterial strains. The presence of such determinants along the food chain underlines the role of food as a source of antimicrobial resistant or virulent bacteria, which could pose a severe hazard to human health. The characterization of AMR and virulence features in artisanal fermented productions has been so far documented (Biswas et al., 2019; Ed-Dra et al., 2019; Pyz-Łukasik et al., 2022; Valenzuela et al., 2009).

From a historical standpoint, for millennia the management of microbial resources has been carried out with unconsciousness of microscopic organisms, by inoculating raw material with a little quantity of matrix from a previous successful fermentation (Brandt, 2014). Following the industrialization of fermented products, "starter cultures" progressively emerged, thus fulfilling the standardized requirements associated with current large-scale fermentations (Capozzi et al., 2017). Basically, starter cultures are preparations of living microorganisms or their resting forms, inoculated in food products to take advantage of their metabolic activity which produces desirable effects in the substrate (Vogel et al., 2011).

One crucial purpose of starter culture technology is to ensure food safety at all stages of fermented food production: domestic, traditional, and industrial. This goal is reached through several biological and metabolic functions, including faster acidification activity, dominance of indigenous microorganism, shorter fermentation time, and decrease of unwanted microbial strains/species and harmful compounds (Chen et al., 2017; De Angelis et al., 2015; Sanchart et al., 2017; Shukla et al., 2014). Notably, starter culture technology is ineffective in and of itself, since the efficiency of each customized strain on a specific feature of the inoculated food matrix is determined by the quality of biotechnological solutions addressing a given unique practical problem. In general, when it comes to safety assurance, it is critical to examine the safety of species/strains as well as the quality/purity of biomass preparation (Capozzi et al., 2017). Moreover, starter cultures express specific traits in relation to differences in the degree of development of various countries. For examples, in developing nations their technological value is strictly connected to the importance of food preservation, yield increase, and food security (Holzapfel, 2002). By contrast, in Western countries they are also developed to pursue individualized nutrition, achieve new health goals, and sustainably enhance shelf life, notably of artisanal, traditional, typical, organic, and biodynamic foods (Capozzi et al., 2017).

Nonetheless, recent social, economic, and environmental developments marked a gradual return to reliance on spontaneous fermentations in the field of traditional, typical, and artisanal fermented foods, including those which benefit from Geographical Indications (PDO, PGI, etc.).

Consistent with this trend, increasing data from scientists and stakeholders contradicts the use of commercial starter cultures with the use of spontaneous fermentation, favouring the latter method in the management of food fermentations (Capozzi et al., 2012). The main driving force which leads towards spontaneous fermentations relies on re-establishing a previously lost content of tradition, typicality, and artisanality (Capozzi et al., 2017). Indeed, many countries now employ starter cultures to realize fermented meat products, although various ancient techniques of manufacturing sausages still exist. Despite the benefits of commercial starters in terms of sanitary safety, their usage may result in the loss of unique traditional features of the finished goods and market approval (Settanni & Moschetti, 2014).

A wide range of microorganisms have been found in conjunction with spontaneous fermentations all around the world, although taking the fermentation process out of control may cause health risks in the context of this diverse microbiota (Cocolin et al., 2016). Spontaneous fermentation may even raise the probability of the irruption of pathogenic microbial strains capable of producing harmful by-products such as mycotoxins, ethyl carbamate, and biogenic amines. Furthermore, the risk of spoiling microbial populations in food matrices could be significant (Cocolin et al., 2016; Russo et al., 2017b). To overcome these hazards, innovative biotechnologies and microbiological methods may be implemented to enhance the safety of spontaneous fermentations. These could be represented by the application of sequence-based molecular technologies (e.g., Whole Genome Sequencing) to investigate the diversity and safety of indigenous microbiota associated with traditional fermented foods, while verifying the legislative safety standard compliance of dominant strains associated with spontaneous fermentation (van Hijum et al., 2013).

All these molecular techniques may provide an excellent solution to assess the presence of strains associated with indigenous microbiota that may represent human health hazards (e.g., presence of genes involved in biogenic amines production and verification of the corresponding phenotype *in vivo*). Given the potential unpredictability of microbial consortia linked with "inoculum enrichment" procedures, implementing these monitoring actions on a regular basis becomes necessary (Capozzi et al., 2017).

Overall, either in the context of using starter cultures or spontaneous fermentation, the early identification of undesired microorganisms (e.g., bacterial foodborne pathogens) is essential to prevent process failure or expensive delays in the fermentation industry and to improve food safety.

2.3. Microbial hazard exposure in dairy and meat artisanal fermented productions

Milk and dairy products have long been key components of worldwide dietary habits and continue to play an important and rising role for both rural and urban populations. These products are generally rich in nutrients, high quality proteins, micronutrients, vitamins and energy-containing fats (Holck et al., 2017; Owusu-Kwarteng et al., 2020). Traditional dairy products and fermented meat embody a

long tradition in Mediterranean countries and are also widely consumed in Europe, where the presence of several local productions, frequently obtained through spontaneous fermentation, is recognized as a remarkable heritage of unexplored microbial biodiversity (Bassi et al., 2022; Salameh et al., 2016).

Food safety hazards are generally linked to any biological, chemical, or physical agent within a food product that has the potential to cause adverse health consequences for consumers (Owusu-Kwarteng et al., 2020). As regard to food safety, fermented foods, including those produced with traditional methods, are typically considered as microbiologically safe, since most pathogens are inhibited or killed by a pH decrease below 4.5, in the acid substrates of raw materials and/or following the synthesis of alcohols and other antimicrobial compounds (Dimidi et al., 2019; Nout et al., 2007). Nevertheless, this may not always be enough to ensure the safety of the food product, as several hazards and potential microbiological risk may be associated to these productions, threatening consumer's health.

In particular, food of animal origin (e.g., milk, meat, together with their secondary products) may be contaminated by pathogenic bacteria during harvest or slaughtering, processing, storage, and packaging, thus representing a risk along the whole food production continuum (Abebe et al., 2020). The latest EU key statistics on zoonoses monitoring and surveillance activities carried out in 2021 by EFSA and ECDC pointed out the magnitude of foodborne outbreaks involving meat and cheese food categories (European Food Safety Authority & European Centre for Disease Prevention and Control, 2022). "Meat and meat products" and "cheese" represented the fifth and sixth food vehicles being cause of strong-evidence foodborne outbreaks, accounting for 20 and 18 outbreaks respectively (Figure 4). Interestingly, "pig meat and product thereof" were included in the top-ten food categories list, being responsible for 15 outbreaks. Moreover, the report estimated that the major causative agent was *Salmonella* for both milk, meat and associated products (12 and 35 outbreaks respectively, Figure 5). Following *Salmonella*, *Staphylococcus aureus* toxins and *Campylobacter* were mainly responsible of milk and milk products outbreaks (n=7 and 2), whereas *Campylobacter* and *Clostridium perfringens* for meat and meat products (n=17 and 13).

As regard to bacterial exposure and transmission along the entire food chain, the production environment plays a distinct role, where animal feces, soil, air, feed, water, equipment, animal hides and people could act as important contributors. For instance, food-producing animals (e.g., cattle, chickens, pigs, and turkeys) are the major reservoirs for many foodborne pathogens such as *Campylobacter* species, non-Typhi serotypes of *Salmonella enterica*, Shiga toxin-producing strains of *Escherichia coli*, and *Listeria monocytogenes* (Heredia & García, 2018).

Another crucial role of food-producing environments is the emergence and spread of AMR. The EFSA Panel on Biological Hazards (BIOHAZ, 2021) identified feed, humans, water, air/dust, soil, wildlife, rodents, arthropods and equipment among terrestrial animal production as sources and transmission routes, where feed and humans reported high evidence. Moreover, several AMR of highest priority for public health, such as carbapenems or extended-spectrum cephalosporin and/or fluoroquinolone-resistant Enterobacterales (including *Salmonella enterica*), fluoroquinolone-resistant *Campylobacter* spp., methicillin-resistant *S. aureus* and glycopeptide-resistant *Enterococcus*

faecium and *E. faecalis* were identified in different sources, at primary and post-harvest level, particularly feces/manure, soil and water.

All these evidence point out that to minimize the food safety risks associated with dairy and meat production chains, especially those lacking product and processes standardization (e.g., artisanal and traditional small-scale facilities), (i) a continuous system of preventive measures, from safety of animal feed, through good farming practices and on-farm controls, to good manufacturing and hygiene practices, (ii) consumers safety awareness, and (iii) proper implementation of food safety management systems throughout the whole food chain, are clearly needed.

Preventive measures based on hygiene practices could be implemented according to good hygiene practice (GHP), good manufacturing practice (GMP) and stringently implementing hazard analysis critical control point (HACCP) along the whole food chain. The HACCP system is so far acknowledged as the most cost-effective strategy of controlling food-borne hazards from 'farm to fork', since it has been created to identify specific hazards and actions to control them to ensure food safety and quality (Panisello et al., 2000). One of the main advantages of the HACCP system is that it focuses attention on possible "problem areas" and requires food facilities to be prepared to deal with problems as soon as they arise. The HACCP system is based on seven principles:

1. Conduct a hazard analysis, which consists in identifying of any hazardous biological, chemical or physical properties in raw materials and processing steps, and then assessing their likely occurrence and potential to cause food to be unsafe for consumption;
2. Identify the critical control points (CCPs), such as steps in the production process at which control can be applied and a food safety hazard can be prevented, eliminated or reduced to acceptable levels;
3. Establish critical limits for preventive measures associated with each identified CCP (e.g., time/temperature, humidity, water activity, pH, salt concentration and chlorine level);
4. Establish CCP monitoring requirements and procedures to assess whether a CCP is under control;
5. Establish corrective actions to be taken when monitoring indicates that a particular CCP is not under control;
6. Establish procedures for verification to confirm that the HACCP system is working effectively;
7. Establish documentation concerning all procedures and records appropriate to these principles and their application (Arvanitoyannis, 2009).

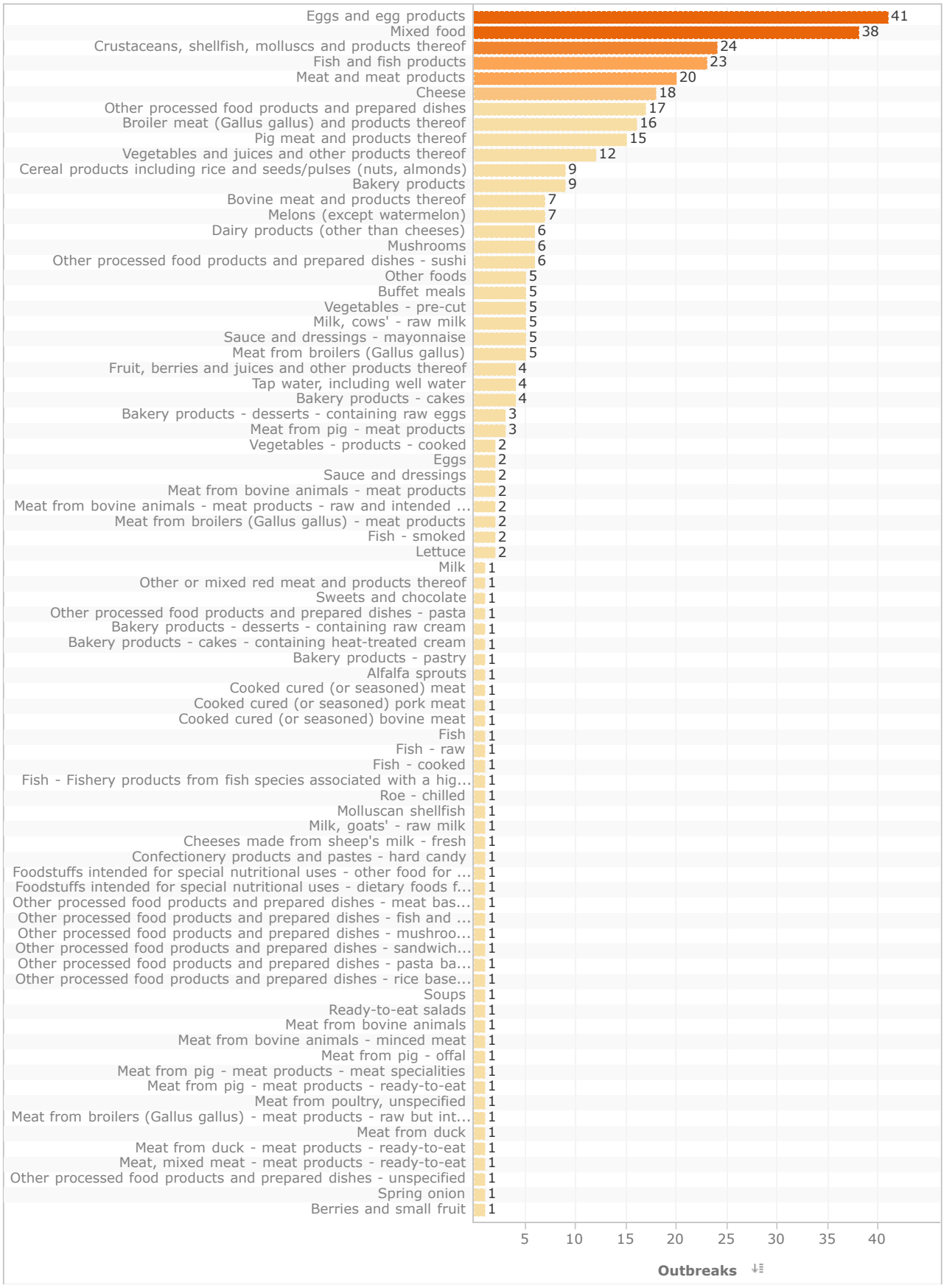

Figure 4: number of strong evidence outbreaks by food vehicle in EU, 2021 (https://www.efsa.europa.eu/en/microstrategy/FBO-dashboard).

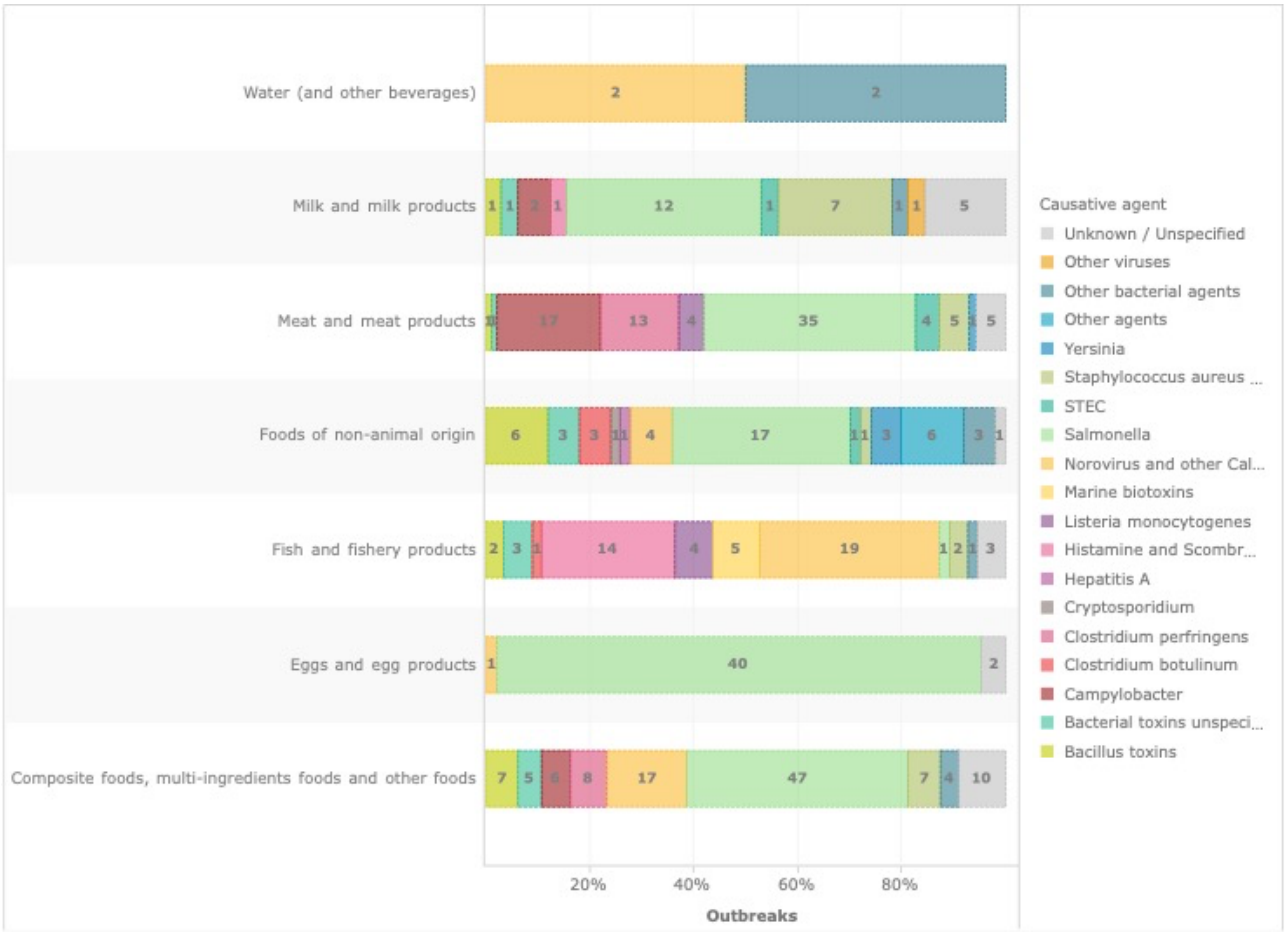

Figure 5: distribution (%) of strong-evidence foodborne outbreaks by food vehicle and by causative agent in EU, 2021 (https://www.efsa.europa.eu/en/microstrategy/FBO-dashboard).

2.3.1. The dairy artisanal chain

Cheese is worldwide perceived as one of the most nutritionally and commercially important fermented dairy products (Chandan et al., 2006). Cheeses' high consumer popularity can be due to their pleasant sensory features, strong nutritional attributes, adaptability, and the possibility of being placed as unique products with novel ingredients, packaging, and selling forms. The sensorial features of a certain type of cheese, such as texture, aroma and flavor, are influenced by specific compounds and molecules (e.g., volatile compounds, free amino acids, phenols, etc.) which are in turn affected by milk's origin and microbiota, starter cultures, environmental bacteria and technological condition of production processes (Tilocca et al., 2020).

Over the last years, an increasing focus has been dedicated to cheese produced according to artisanal and traditional methods. The main differences between artisanal and industrial cheese rely on milk's quality and nutritional value, where its composition is typically reflected by seasonality, as well as the absence of additives employed during cheese-making processes (Cirne et al., 2019).

In general, cheese manufacturing involves several technological processes, including the coagulation of milk proteins resulted from the addition of rennet and other coagulation agents, followed by the curd formation and whey draining. Ripening is the most important technological step as it embraces

a deluge of biochemical reactions generated by a wide microbial consortium, including the organoleptic attributes perceived by consumers (Khattab et al., 2019).

Regarding food safety, in recent decades cheese has been connected to several foodborne outbreaks in Europe and worldwide (André et al., 2008; Dzieciol et al., 2016; Kousta et al., 2010; Lee et al., 2012; Martinez-Rios & Dalgaard, 2018; Rückerl et al., 2014; Schön et al., 2016). Foodborne pathogens have been mainly recovered in soft cheeses from raw milk, whilst some outbreaks are also described in association to pasteurized-milk cheeses (Possas et al., 2021).

Cheese contamination by bacterial pathogens may occur along the entire manufacturing processing, from cheese-making to ripening, until the end of the storage, as a result of direct or cross-contamination events (Kousta et al., 2010). Several reports have identified foodborne pathogens in raw milk from farm bulk tanks and dairy silos, indicating that raw milk might represent a source for cheese contamination (Van Kessel et al., 2004). *Staphylococcus aureus* is one of the major contaminants of raw milk, being the cause of mammary glands infections with clinical or subclinical staphylococcal mastitis, whereas *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* could contaminate raw milk from the agricultural environment (e.g., feces) (Kousta et al., 2010; Van Kessel et al., 2004). Therefore, effective intervention strategies should be implemented to reduce pathogen accumulation, survival, and transmission in the farm environment, including animal and waste management, water treatment, excellent sanitary conditions during milking, and mastitis prevention (Fox, 1999).

Beyond good farm practices, pasteurization is the crucial technological process aimed at killing bacterial pathogens, except for spore-forming bacteria, thus expecting absence of pathogenic strains along further processes. In addition, low water activity (Aw) and pH combined with competition with starter cultures are common hurdles employed to hamper survival and growth of undesirable and pathogenic bacteria (Morgan et al., 2001). On the other hand, if abuse temperatures occur in raw milk during transport or storage at the processing plant, psychrotrophic pathogenic bacteria such as *L. monocytogenes* might grow and multiplicate to critical levels, thus generating the ineffectiveness of low temperatures treatment (60-63°C) commonly used in cheese production (D'Aoust, 1989). At the processing plant, various sources could act as a vehicle for pathogens contamination, such as ingredients (e.g., starter cultures, brine), floor and packaging material, cheese vat, cheese cloth and curd cutting knife, cold room and production room air (Temelli et al., 2006).

Several studies showed the isolation of *L. monocytogenes* from both food-contact and non food-contact surfaces in cheeses' facilities. In particular, *L. monocytogenes* strains have been found in the environment contaminating drains and floors (Kabuki et al., 2004) as well as packaging equipment (Kells & Gilmour, 2004), as a results of cleaning failure. Another study revealed storage coolers as vehicle for the dissemination of *L. monocytogenes* in cheese from pasteurized milk (Brito et al., 2008). When *L. monocytogenes* colonize processing equipment, it can form biofilm and thus persist overtime in food-contact surfaces (Møretrø & Langsrud, 2004). Thus, both food-contact surfaces and the environment of dairy facilities might be possible sources of contamination of the final product. *L. monocytogenes* contaminating cheese is alarming due to the capacity of this pathogen to growth or extended survival under refrigerated settings, depending on the cheese type (Kousta et al., 2010).

Another source responsible of cross-contamination at the cheese processing plant is worker's hands, which have been identified as a potential vehicle for the dissemination of *S. aureus,* a common inhabitants of the human skin and nasal cavity (Lee et al., 2012). Moreover, *Salmonella* spp. has also been identified as a prevalent cause of foodborne outbreaks connected to cheese products and associated with the primary manufacturing and processing environment (Robinson et al., 2020).
Other bacteria commonly found in cheese are represented by coliforms, members of the family *Enterobacteriaceae*, which are generally used as indicators of process hygiene (Gelbíčová et al., 2021; Martin et al., 2016).
Common coliform genera in raw milk include *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella* (Jayarao & Wang, 1999). Various outbreaks associated with the consumption of raw-milk cheeses caused by Shiga toxin-producing *E. coli* (STEC) have been documented worldwide in the last decade (Currie et al., 2018; Jones et al., 2019; McCollum et al., 2012). In addition, *Klebsiella pneumoniae* and *Klebsiella oxytoca* strains have found to be persistent between the production environment and the personnel in a processed cheese plant (Gelbíčová et al., 2021) and from a traditional Turkish cheese (Uraz et al., 2008). Although not recognized as a major foodborne pathogen, member of the *Klebsiella* genus represent a significant public health problem worldwide, being among the most common causes of both hospital and community-associated infections due to their virulence factors and/or multiresistance to antibiotics. Being increasingly recovered from several food products categories (Hu et al., 2021), the presence of *Klebsiella* spp. among cheese productions, suggests the importance of further describing its occurrence in food and related environment, not only as a hygiene indicator, but also concerning its antibiotic resistance and virulence potentials.
With respect to the safety and public health issues connected to processed cheeses, especially those produced at the artisanal level, it is necessary to apply intervention strategies to obstacle the development of contaminating microflora. These include preventing raw milk contamination through good farm practices and mastitis control programs at the farm level. Likewise, to prevent *L. monocytogenes* from colonizing the processing environment, plant structure and equipment should be designed to be hygienic, promote appropriate working routines and provide an effective sanitation process (Wirtanen et al., 2000).

2.3.2. The fermented meat artisanal chain

Fermented meats, such as dry fermented sausages, represent a food category with high popularity in European food markets (Belleggia et al., 2020; Leroy et al., 2018). A wide range of products are produced and distributed according to differences in raw ingredients, formulas, and production techniques resulting from the habits and customs of different nations and regions. Artisanal dry fermented sausages are generally produced from raw meat mixed with fat, curing salt, and spices, and processed without the addition of starter cultures (Corbière Morot-Bizot et al., 2006).
Pork meat represent the most commonly meat origin employed in dry fermented sausages in Europe, followed by beef or horse meat (Coloretti et al., 2019; Geeraerts et al., 2019; Settanni et al., 2020).

Among European dry fermented sausages, salami, which are traditionally made with pork meat and fat, are widely popular, especially those produced according to artisanal processes (Talon et al., 2007). From a technological perspective, traditional artisanal salami are commonly realized without employing nitrate or nitrite, which is combined to the absence of starter cultures (Settanni et al., 2020).

In the fermented meat food industry, nitrate and nitrite have long been used to preserve meat products from spoilage and to extend their shelf life. Moreover, they also contribute to the perceived organoleptic attributes connected to colour and flavour (Lopez et al., 2021). The absence of these additives in artisanal productions contributes to their increasing appreciation among consumers, since they are perceived as more "natural" compared to the industrial ones (Settanni & Moschetti, 2014).

After meat mixture preparation, salt, species, and other natural preservatives are added, and all ingredients are stuffed into casing, thus initiating a series of complex microbiological reactions involving fermentation and ripening processes (Cocolin et al., 2009; Corbière Morot-Bizot et al., 2006). Because no starter cultures are introduced, these products are distinguished by the occurrence of spontaneous fermentations by indigenous microorganisms typically found in raw materials. Notably, their remarkable technological characteristics confer specific and original flavors to typical regional fermented sausages, allowing manufacturer to diversify the market (Franciosa et al., 2018).

Nevertheless, these microorganisms should replace specific technological properties belonging to starter cultures, including the ability to quickly growth at high levels, and prevent the development of undesirable spoilage and harmful bacteria (Holzapfel, 2002; Montel et al., 2014). Consistent with these technological requisites, raw meat contains all necessary nutrients in accessible form, as well as a nearly neutral pH and high Aw, thus supporting the growth of all microbial groups of food interest (Settanni et al., 2020).

Microorganisms identified from the indigenous bacteria of spontaneously fermented sausages mainly belong to the LAB group, as well as the *Staphylococcus* and *Kocuria* species (Corbière Morot-Bizot et al., 2006).

The LAB are the primary diversifiers of matrix acidification because they assure product stability primarily by forming lactic acid, which inhibits pathogen development. Among LAB, *Lactobacillus sakei* and, to a lesser extent, *Lactobacillus curvatus* and *Leuconostoc* spp. are the most frequently found (Comi et al., 2020; Wang et al., 2019). While *L. sakei* prevalence is typically not related with specific quality or safety problems, *Leuconostoc* spp. and *L. curvatus* can occasionally produce harmful concentrations of biogenic amines (Barbieri et al., 2019; Li et al., 2018). Because these compounds are neurotoxic and have been connected to food poisoning cases, high amounts constitute a public health concern (Torović et al., 2020).

Among *Staphylococcus* spp., coagulase-negative staphylococci are characterized by a nitrate reductase activity, thus contributing to color development, and their amino and fatty acid degradations improve sensory qualities of fermented sausages (Talon et al., 1999). Although *Staphylococcus xylosus*, *Staphylococcus saprophyticus*, and *Staphylococcus equorum* are generally the most frequent species in most natural fermentation processes, there is a significant species variety in staphylococci communities. Among additional encountered species: *Staphylococcus warneri*, *Staphylococcus*

epidermidis, *Staphylococcus sciuri* and *Staphylococcus succinus* (Barbieri et al., 2019; Comi et al., 2020; Cruxen et al., 2019; Li et al., 2018; Wang et al., 2019).

Because salami do not undergo heating treatments throughout the manufacturing process or before consumption, their production must be carried out in respect of high standard Good Manufacturing Practices and Good Hygienic Practices criteria. Moreover, environmental conditions characterized by high acidity, high salinity and low Aw, combined with the persistence of natural competing microbial populations should ensure the microbiological safety of final products. Nevertheless, some pathogens might be able to survive or grow even in presence of adverse environmental conditions, either by directly as raw contaminants (e.g., raw meat) or through cross-contamination from equipment or personnel during processing or at retail (Holck et al., 2017; Moore, 2004).

Among foodborne pathogens, *Salmonella* represents one of the most common cause of outbreaks associated to fermented sausage consumption, where meat ingredients have been reported as a critical vehicle for pathogens contamination. Moreover, the highest number of outbreaks is linked to fermented sausages from pork meat contaminated with *Salmonella Typhimurium*, even though other serovars (e.g., Montevideo, Goldcoast) have also been noticed (Bremer et al., 2004; Gossner et al., 2012; Kuhn et al., 2011).

Another potential hazard during fermented sausages manufacturing is *Listeria monocytogenes*, which is distinguished from the severity of the disease, the extensive diffusion in processing equipment and environments, and the hypothetical capability of the food product ecology to sustain its growth (Ferreira et al., 2011; Gounadaki et al., 2008; Thévenot et al., 2005). *L. monocytogenes* may contaminate raw materials at many stages of the production process, such as from slaughterhouse environment or during/post-processing via unclean surfaces or the personnel (Chasseignaux et al., 2002; Colak et al., 2007; Thévenot et al., 2005). After processing plant colonization, *L. monocytogenes* may survive and persist in the processing environment over time thanks to the ability of developing in biofilms (Meloni, 2015). Generally, a higher recovering of these pathogens has been observed in pork meat (Thévenot et al., 2006), although rare case of critical listeriosis outbreaks have been documented in fermented sausages (Meloni, 2015).

As respect to the *Staphylococcus* genus, few cases of *S. aureus* food poisoning have been associated to fermented sausages. Although the low incidence, *S. aureus* presence in such productions should be monitored due to their ability of survive in acidic or alkaline medium (pH = 4.5–8.0), salt concentrations up to 15%, and low water ($a_w \geq 0.83$) (Bryan, 1988; Hennekinne et al., 2012; Kérouanton et al., 2007; Wieneke et al., 2009).

Enterobacteriaceae could also be found in the sausage mass because of contamination occurred either during slaughtering procedures, being commensal of the gastrointestinal tract of animals, or from utensils used during processing (Fernández-López *et al*., 2008). Enterobacteria are generally undesired in food fermentations as some species can be pathogenic and/or linked with the presence of biogenic amines (Cruxen et al., 2019; Sun et al., 2019; Van Reckem et al., 2019).

Among this family, STEC O157:H7 has been associated with foodborne outbreaks related to the consumption of dry-fermented salami (Conedera et al., 2007; Woodward et al., 2001). Moreover, other reports described an extensive pathogen survival in dry fermented sausages (Calicioglu et al.,

2002; Heir et al., 2010; Nissen & Holck, 1998). Compared to other serotypes, O157:H7 are characterized by an improved tolerance to acids and low pH, which is reached at the end of the ripening process, suggesting considerable challenges in looking for effective strategies to STEC eradication (Arnold & Kaspar, 1995; Bergholz & Whittam, 2007).
Regarding other Enterobacteriaceae, *Klebsiella* spp. has been isolated in Europe before the ripening process of pork sausages (Roig-Sagues et al., 1996) and in fermented sausages produced without starter addition (Barbieri et al., 2021).
Overall, whilst traditional manufacturing processes provides an intriguing occasion for small-scale operators to diversify their products, traditional and empirical processes knowledge should be crucial to ensure products food safety (Cocolin et al., 2016).
Besides preventive hygienic measures applied at slaughterhouses, food products safety is mainly based on hurdle technology and rely on a combination of many preservation procedures. Beyond LAB activity and Aw reduction, additional factors such as processing temperature and pH, the use of preservatives, smoking, spices, herbs and wine can all have a significant impact on pathogen development or survival (Patarata et al., 2020).

3. Role of Whole Genome Sequencing in food safety microbiology

3.1. The rise of High Throughput Sequencing technologies

Sanger et al. (1977) released the first genome sequencing of the bacteriophage phiX174 in 1977, opening a new avenue into the genomics era. Hereafter, the first bacterial genome (*Haemophilus influenzae*) was sequenced over 20 years later using the same process, which included DNA extraction, random shearing, cloning into plasmid vectors, *E. coli* transformation and propagation, plasmid DNA extraction, and Sanger sequencing (Fleischmann et al., 1995). The latter consisted in an advanced sequencing technology used for small DNA fragments around few 1000 bp yet revealing as time consuming and more processes demanding whether applied for longer stretches of DNA.
The introduction of next-generation sequencing (NGS) technology in the early 2000s radically transformed microbial genomics as well as the way DNA sequencing could be used routinely in food safety and public health. This new technique coupled microfabrication breakthroughs with an innovative new sequencing process to create enormous volumes of nucleic acid sequencing data cheaply and quickly (Taboada et al., 2017). With the NGS approach, an entire bacterial genome can be sequenced in small random fragments (100 to several 1000 bp) multiple times in a single reaction, also known as High-Throughput Sequencing (HTS) or massive parallel sequencing. The full DNA sequence is then determined *in silico* by connecting fragments with overlapping sequences using sequence assembly software (Margulies et al., 2005; Vincent et al., 2017). With the advent of HTS technology, it became feasible to create thousands to millions of sequences in a single run and in a matter of days for a few hundred dollars, reducing both time and money when compared to previous culture-independent approaches (Loman et al., 2012; Mayo et al., 2014). These technologies also involve a safer bench activity with less exposure to hazardous chemicals, such as electrophoresis

(Ercolini, 2013). Furthermore, the introduction of benchtop technologies has increased the popularity of HTS by allowing numerous research organizations to undertake genome sequencing in their own facilities (Edwards & Holt, 2013).

Since Roche's first HTS platform (the 454 platform, now discontinued) was released to the market in 2004, several platforms from different companies have been developed, resulting in continuous technological improvements and significant reductions in sequencing costs. Indeed, the cost per sequenced Mbp was 5292$ in September 2001, then followed a dramatically drop in 2008 (from 397$ in October 2007 to 3.81$ in October 2008), as sequencing centers evolved from Sanger sequencing to HTS. Nowadays, the cost per sequenced Mbp is less than 0.012$, enabling the acquisition of high-quality bacterial genomes for less than 100€ per genome using an Illumina MiSeq technology (Edwards & Holt, 2013).

Over the next decade, two major NGS technologies emerged: short read and long read technologies, which were defined principally by the length of the produced sequence segment ("read"). Short read technologies, such as those used in the Illumina and Life Technologies platform lines, yield read lengths ranging from 100 to 600 bp with low per-base error rates (usually less than 1%). These methods are commonly used to construct draft genome sequences with high accuracy and coverage (>95% for an average bacterial genome) (Goodwin et al., 2016). Short read platforms include “sequencing by oligonucleotide ligation and detection” of SOLiD (Life Technologies) in 2007 or “sequencing by synthesis and semiconduction” of Ion Torrent (acquired by Life Technologies), and “sequencing by cluster synthesis” of Solexa (later Illumina) in its first GAII equipment that appeared in 2006, and subsequent sequencers, such as the MiSeq marketed in 2011 and the more powerful sequencers NextSeq, HiSeq, or NovaSeq (Quijada et al., 2020).

The benchtop MiSeq from Illumina is one of the most used HTS technologies for microbiology research (Pallen, 2016). Originally launched in 2011, the MiSeq quickly gained popularity because of its low error rate and simple workflow (Loman et al., 2012). Following various steps to create pure DNA libraries from extracted nucleic acids, the MiSeq device generates millions of reads up to 300 bp in a single run ($15 Gbp/run) (Mayo et al., 2014). Moreover, this technology enables paired-end sequencing, which involves sequencing from both ends of a DNA fragment. This function improves the accuracy of genome assembly in microbial genomics and is commonly used in microbial taxonomy because it facilitates the formation of fragments that are almost 600 bp long owing to read overlapping. Although the length of the sequenced taxonomic target is an essential feature for containing enough nucleotide variation to discriminate between species, it also represents the major weakness of Illumina sequencing systems (Arredondo-Alonso et al., 2017; Soergel et al., 2012). To address this issue, some sequencing technologies have been created, such as Pacific Biosciences' PacBio RS platform, which is based on single-molecule sequencing and provides sequence lengths of up to 15,000bp (Loman et al., 2012). However, because of its sequencing/instrumental costs, Illumina technology is presently the sequencing platform most widely used (Pallen, 2016).

Besides short read platforms, longer read technologies have been developed, encompassing third generation of sequencers that use single-molecule sequencing (single molecule real-time, SMRT), such as the portable MinION from Oxford Nanopore Technologies (Quijada et al., 2020). As PacBio,

MinION use single-molecule sequencing to generate read lengths ranging from 1,000 to over 100,000 bp, although it still have rather high error rates (15-30%) (Goodwin et al., 2016). Long read technologies allow to create almost complete genomes consisting of few sequence fragments called "scaffolds". Combined with short read technologies, high quality draft genomes can be efficiently reconstructed. It is also feasible, although expensive, to build a high-quality, "full" bacterial genome with only the implementation of long-read technology. Long read sequencing methods have other specialized uses that may be relevant for future food science applications, as detailed below (Taboada et al., 2017).

3.2. Analytical post-sequencing approaches for microbial typing

Over three decades ago, the introduction of PCR technology into the analytical microbiology laboratory shifted the basis for bacterial identification from a phenotypic standard to one based on genotype (Blais et al., 2012; Huszczynski et al., 2013). The advent of molecular biology, drove the way to molecular typing methods relying on specific target DNA sequences, such as segments that are unique to a certain species or subtype. In general, molecular subtyping methods can be classified by using amplification, restriction digestion, or DNA sequencing, depending on the pathogen under research (Ronholm et al., 2016). Thus, most molecular typing procedures are based on changes in target DNA sequences.

Molecular typing methods share common drawbacks, including time-consuming processes and lengthy analysis timeframes (Wei & Zhao, 2021). Additionally, typing technologies such as PFGE and MLVA reveal just a small portion of the information stored inside a foodborne pathogen's genome, limiting the capacity to distinguish outbreak-related pathogen strains from unrelated, sporadically circulating variants (Taboada et al., 2017).

Recent advances in the ability to sequence a full bacterial genome in a fast and cost-effective way have overcome these obstacles, by giving information with previously unheard-of accuracy about a pathogen at the DNA and gene levels (Aarestrup et al., 2012; Allard et al., 2018). The method is known as whole genome sequencing (WGS). Unlike traditional molecular typing tools, WGS may theoretically unveil the whole genome of a specific microbial pathogen, allowing for the differentiation of strains that differ by a single nucleotide or gene and therefore perfectly representing bacterial genomic polymorphism (Taboada et al., 2017).

The advancement of HTS platforms and technologies, as well as the reduction in related running time and costs, have drastically transformed how food microorganisms are studied, opening new avenues for undertaking extensive research on foodborne bacteria. Indeed, the huge amount of data created by HTS platforms provides more information than ever, allowing us to better monitor and improve our understanding of undesired microbes associated to the food chain, such as spoilers or pathogens. Among HTS, WGS has demonstrated its potential usefulness in elucidating the genetic content of food chain-associated microorganisms (Logares et al., 2012; Quijada et al., 2020).

Following the WGS method, bacteria are firstly collected from the sample, then DNA is extracted from the pure culture, prior to library creation and HTS, and sequence reads are finally generated

(Quijada et al., 2020). WGS offers ultimate resolution for subtyping of bacteria at strain level (Franz et al., 2016). Genomic subtyping allows to identify bacterial strains at a much deeper phylogenetic resolution than species-level, and to discriminate clonally related strains in outbreaks or contaminations, which is critical in food safety and human health (Quijada et al., 2020).

WGS is being introduced in the field of public health, complementing and, in some cases, replacing most current identification and characterization methods in the microbiology laboratory, such as serotyping, virulence profiling, AMR determination, and previous molecular typing methods, providing faster and more precise results. Indeed, WGS provides numerous benefits over traditional molecular typing techniques, including high resolution, high repeatability, strong comparability, easy operation, and rapidity (Jagadeesan et al., 2019; Wei & Zhao, 2021).

In the EU, great efforts have been made to develop integrated platforms for sharing epidemiological as well as subtyping information (e.g., the INNUENDO platform, https://innuendo.readthedocs.io/en/latest/). In particular, the great advantage of sharing sequence data (instead of strains or DNA) useful not only for typing but also for strain characterization (e.g., AMR, virulence), allowed outbreaks to be discovered sooner and hence resolved faster. For example, in the year following WGS implementation for prospective assessment surveillance of listeriosis in the United States, more and smaller outbreaks were detected, outbreaks were detected earlier, outbreak sources were identified more frequently, and the total number of outbreak-related cases identified increased (Jackson et al., 2016).

At the EU level, latest key statistics from 2021 data outlined similar trends in *L. monocytogenes* outbreaks detection. Consistent with this, EFSA reports suggested that increased detection of small listeriosis outbreaks in the population was connected to more sensitive laboratory-based surveillance and, in particular, WGS (European Food Safety Authority & European Centre for Disease Prevention and Control, 2022).

Another advantage is that WGS can yield unprecedented levels of information about the presence of virulence and other marker genes relevant to the identification and risk characterization of food isolates, whether in the context of outbreak investigations or information gathering in the course of research (Carrillo & Blais, 2021). To address this outcome, WGS analysis is currently supplanting and replacing numerous traditional microbiological investigations to identify and characterize bacteria, such as serotyping, AMR, and virulence profiling, in a single quick and cost-effective WGS workflow (Allard et al., 2016; Carleton & Gerner-Smidt, 2016).

Overall, WGS has increased the precision of surveillance, allowing for faster and more efficient decision making in the prevention and response to foodborne diseases (Brown et al., 2019).

On the other side, turnaround time, empirical knowledge for genotype-phenotype correlation and data analysis are among the most concerning barriers of WGS. In general, there is a limited tolerance for the WGS turnaround time to be longer than conventional approach when sequencing is being utilized to provide an interpretation that could be produced using more traditional methodology. Hence, turnaround time must be as quick as established procedures, or possibly faster, in order to have a substantial impact on clinical decision making. Concerning genotype-phenotype correlation, only by experimentally building the knowledge base will it be possible to overcome the barrier of the

connection of high-throughput sequencing data and the related phenotype. For instance, the clinical impact of virulence genes ought to be assessed to meaningfully interpret their relevance (Rhoads, 2018).

However, the frequent use of WGS necessitates the deployment of low-cost, user-friendly solutions that may be employed on-site by staff who are not experts in large data management (Hyeon et al., 2018). Extraction of useful information from HTS data, on the other hand, necessitates advanced computing tools and abilities, which is the fundamental bottleneck of HTS (Carriço et al., 2018). Nonetheless, the high discriminatory power of WGS compared to traditional molecular typing tools is well established, and WGS has the potential to become a one-stop-shop solution for routine bacterial analysis. It indeed improves multiple steps in traditional microbiology diagnosis by providing highly accurate, in-depth analysis in a short period of time (Moran-Gilad, 2017).

WGS of microbial pathogens has now been implemented in at least four nations for prospective monitoring of bacterial foodborne pathogens: the United Kingdom, Denmark, France, and the United States (Allard et al., 2016; Ashton et al., 2016; Jackson et al., 2016; Kvistholm Jensen et al., 2016; Moura et al., 2016). In the United States, WGS is rapidly being implemented by food regulatory and public health authorities to support in the discovery, investigation, and control of foodborne bacterial outbreaks, as well as food regulatory and other operations in support of food safety (Brown et al., 2019).

The widespread use of bench-top platforms, like as the MiSeq, has resulted in an unprecedented number of sequenced genomes, which are shared through services such as the National Center for Biotechnology Information (NCBI) and are available for other researchers to use and compare (Edwards & Holt, 2013).

Public databases, such as NCBI, harbour massive amounts of WGS data for diverse bacteria, including the majority of those of relevance to the food microbiology laboratory, representing a valuable resource reflecting a vast diversity of microorganisms collected from across the world. At the time of writing, the NCBI Pathogen Detection database has 279072 *E. coli*/*Shigella*, 498997 *S. enterica*, 81379 *Campilobacter jejuni* and 54105 *L. monocytogenes* genomes (https://www.ncbi.nlm.nih.gov/pathogens/).

These databases are free to use and include a variety of tools for genomic study. As a drawback, they miss key metadata, which makes source identification and comparison difficult, as well as potential sequence data quality concerns (e.g., incomplete or contaminated sequence) (Carrillo & Blais, 2021). Data sharing is also important in retrospective research, which may be readily conducted in older datasets when new genes are discovered (Schürch & van Schaik, 2017). As a result, this technology is appropriate for use in national and international monitoring systems for food safety and public health. Aside from increasing outbreak identification and response, it will most certainly increase our understanding of the epidemiology of many infectious illnesses in the next years (Brown et al., 2019).

3.2.1. High resolution typing

As previously highlighted, the highly discriminative power of WGS enables the comparison of genetic relatedness between bacteria even on a sub-species level. In this context, two analytical post-sequencing strategies are used to decipher and compare raw nucleotide data of a full sequence of bacterial genomes, such as base by base (single-nucleotide polymorphism [SNP] analysis) or gene to gene (multilocus sequence typing [MLST]). Both methods may be used to trace clinical isolates back to environmental or contaminated food sources, or to cluster food and environmental isolates over time and across spaces to detect outbreaks (Brown et al., 2019).

Briefly, a SNP is defined as a nucleotide change at a specific place in the genome of a test strain compared to the sequence of a reference strain, following a genetic mutation event. SNPs could be found across the genome, encompassing both coding and noncoding areas. The selection of a reference genome for SNP identification is critical to obtain useful results and should be adapted to the specific circumstance. For instance, in the context of an outbreak setting, a reference closely related to an isolate involved in the outbreak should provide the most accurate assessment of the SNP differences. If DNA segments are only found in the reference or test isolates, they will be excluded from the analysis, reducing the information to study. Moreover, since SNPs found on mobile elements such as phages, insertions or deletions and plasmids may not be phylogenetically significant, they are frequently filtered out in the final analysis to reduce noise from the epidemiological study. Once all the test isolates' SNPs have been identified, the SNP profiles are all together submitted to pairwise comparison and generally visualized using a phylogenetic tree. Thus, by making use of nearly all of the genetic information from a genome/strain, SNP analysis provides the potentially maximum degree of discriminatory power attainable for strain phylogeny reconstruction (Brown et al., 2019).

By contrast, the MLST approach evaluates sequence changes in the coding regions of genes, defined as "loci", by detecting any changes, including SNPs, insertions or deletions, and recombination (Maiden et al., 2013). MLST is a common bacterial typing approach that has typically been performed using classic Sanger sequencing, in which few conserved genes (so called housekeeping genes) per isolate are sequenced from end to end. With the decrease in HTS costs, collecting a draft genome with complete genomic information is now significantly cheaper than sequencing the MLST housekeeping genes separately (Ronholm et al., 2016). This method allowed to extend the MLST concept at the genome scale and is extremely adaptable since the quantity and nature of the genes evaluated may be adapted to each specific circumstance and genome. Currently, several levels of discrimination are available based on the gene-by-gene approach, including (i) seven housekeeping gene (7-gene) MLST, (ii) core genome (cg) MLST, which examines genes present in nearly all strains of a given species, and (iii) whole genome (wg) MLST, which analyses genes present in almost all strains of a given species.

For bacterial strain typing and nomenclatural purposes, the MLST is based on indexing the allele variation among 5-7 housekeeping genes into allele profiles and the translation of such profiles into sequence types (STs). This allows to separate isolates of a species in a broad phylogenetic context,

and represent them through epidemiologically relevant groups such as clonal complexes (Brown et al., 2019; Kotetishvili et al., 2002).

While MLST relies on the presence of SNPs in a few conserved genes to assign a ST to a given genome, WGS enable comparison of the entire pangenome and the development of extended MLST frameworks, such as core (cgMLST) and whole genome (wgMLST), (de Been et al., 2015; Kohl et al., 2014). The cgMLST offers the most comprehensive and informative phylogenetic distinction of a species. Furthermore, when the accessory genome (e.g., genes do not present in all isolates) is also analyzed, the wgMLST can provide even more discrimination than the cgMLST, and be useful for cluster investigations to discriminate between closely related isolates, though observed differences may not reflect true phylogeny.

All approaches based on MLST involve the comparison of genes found in test strains to a reference database of genes that includes all known gene variations known as "alleles". Unlike SNP analyses, database of loci and alleles from several strains, rather than a single reference genome, constitute the MLST reference schemes. An identifier is attributed to each unique allele sequence, and genomes are compared using the allele profiles distance. Similar to core SNPs analysis, cgMLST frequently filtered out mobile elements in the final analysis, since the changes identified are not necessarily epidemiologically meaningful. However, MLST cannot detect differences in noncoding regions (e.g., between genes), resulting in a lower resolution compared to SNP analysis.

MLST and cgMLST schemas are publicly available and currently curated for main bacterial species of clinical interest, and their usage is highly recommended for strain subtyping (Figure 6). Available schemes are hosted in different webplatforms and institutions. For instance, the 1748-loci scheme of *L. monocytogenes* was developed by Institut Pasteur, France and is accessible at BIGSdb.Pasteur.fr (http://bigsdb.pasteur.fr/listeria), while the *Campylobacter* schema from the University of Oxford, United Kingdom is accessible at PubMLST.org (https://pubmlst.org/ campylobacter), and the *Salmonella* and *E. coli* schemas from University of Warwick, United Kingdom are available through the Enterobase platform (https://enterobase.warwick.ac.uk/species/index/senterica and https://enterobase.warwick.ac.uk/species/index/ecoli). For other foodborne bacterial species, public cg/wg MLST schemes are very limited.

cgMLST and Genomic Reference databases for key food pathogens.

Pathogen	DB location	Hosted by	Validation
Listeria monocytogenes	http://bigsdb.pasteur.fr/listeria/	Institut Pasteur, FR	Moura et al. (2016)
Salmonella	https://enterobase.warwick.ac.uk/species/index/senterica	Warwick University, UK	–
Escherichia/Shigella	https://enterobase.warwick.ac.uk/species/index/ecoli	Warwick University, UK	–
Yersinia	https://enterobase.warwick.ac.uk/species/index/yersinia	Warwick University, UK	–
Campylobacter	https://pubmlst.org/campylobacter/	University of Oxford, UK	–

Figure 6: description of cgMLST schemes referred to key foodborne pathogens (Jagadeesan et al., 2019).

3.2.2. Comparative genomics for surveillance of bacterial strains

Both SNPs and gene-by-gene approaches can be at the basis of comparative genomic studies. The choice of the method would depend by the end-demands of the user along with the epidemiological setting and the targeted bacterial population (clonal *vs* panmictic). SNP-based methods are preferable

to analyse highly clonal populations and are likely less effective for other species. While either SNP or gene-by-gene approaches can be applied to investigate a fixed number of strains linked to a specific contamination event, cgMLST profiles generated with consistent methods (i.e., same reference scheme database and allele caller) can be compared consistently across labs. This makes cgMLST more suitable if multiple users need to systematically analyse every new isolate added to a common database, such as in an outbreak surveillance network (Jagadeesan et al., 2019). In addition, MLST-based methods are highly standardizable while establishing a standard set of SNPs across a population is not possible. Although some resolution is sacrificed with cg/wgMLST compared to SNP analysis, allele profiles can be used to infer phylogenetic trees (Figure 7) (Chen et al., 2017; Cunningham et al., 2017; Katz et al., 2017).

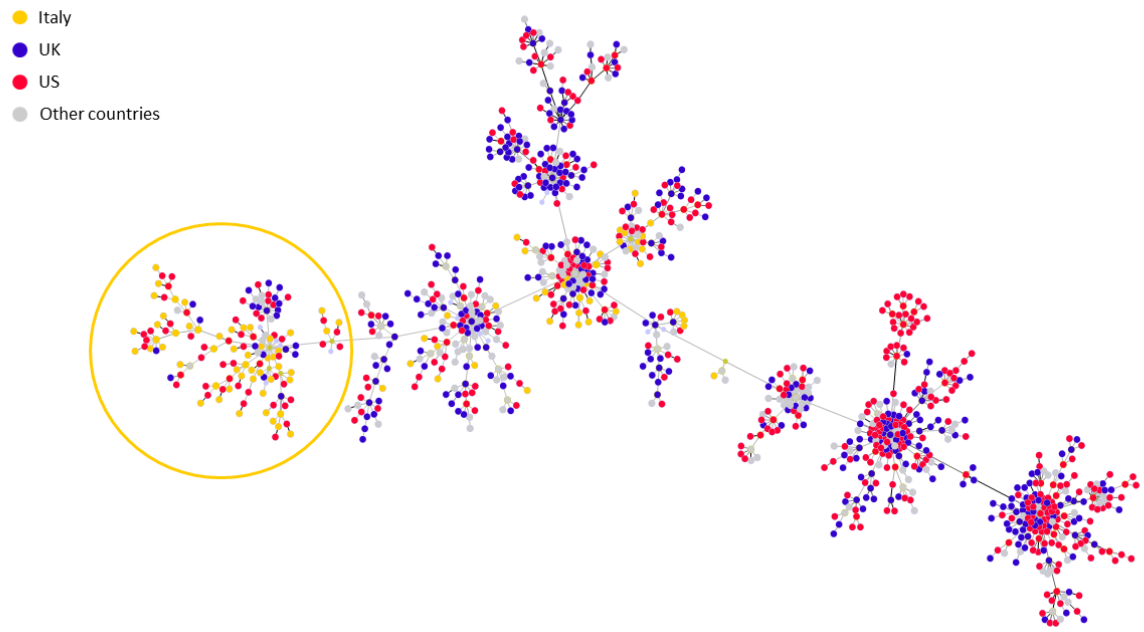

Figure 7: example of Minimum Spanning Tree of 1,326 genomes of *Salmonella enterica* ser. Typhimurium based on a 3,591 loci cgMLST schema. Adapted from Palma et al., (2018).

Altogether, SNP and gene-by-gene approaches evaluate genetic variations in slightly distinct ways, suggesting to employ both when one method alone does not yield clear-cut answer or when greater evidence for an association between isolates (e.g., to confirm the source of an outbreak) has to be investigated (Jagadeesan et al., 2019).

To provide a robust and efficient tool for rapid detection of multi-country food-borne outbreaks with the ultimate purpose of serving public health interests and protecting European consumers, EFSA has implemented the "EFSA One Health WGS System". This system interoperates with the ECDC

Molecular Typing system by exchanging (WGS) data, including cgMLST profiles and minimum metadata, of *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli*, including STEC, isolates from human and non-human origins (e.g., food, feed, animals, and the farm and food processing environment isolates) (European Food Safety Authority, 2022).

The genetic variation found by SNP or gene-by-gene analysis, referred to as the number of SNP/allele differences in a pair-wise comparison, can be used to infer phylogenetic trees, providing information on the evolutionary history and phylogenetic connections of isolates (Figure 8). Phylogenetic trees display the estimated evolutionary model of a set of isolates as a succession of branches from the root or common ancestor. Different models can be used to infer phylogenies including parsimony, maximum likelihood (ML), and Bayesian or distance approaches such as Neighbour Joining (NJ). Whatever model is used to infer a tree, isolates that will be grouped together in so called clusters will be more closely linked than isolates found sparse in the tree (Jagadeesan et al., 2019).

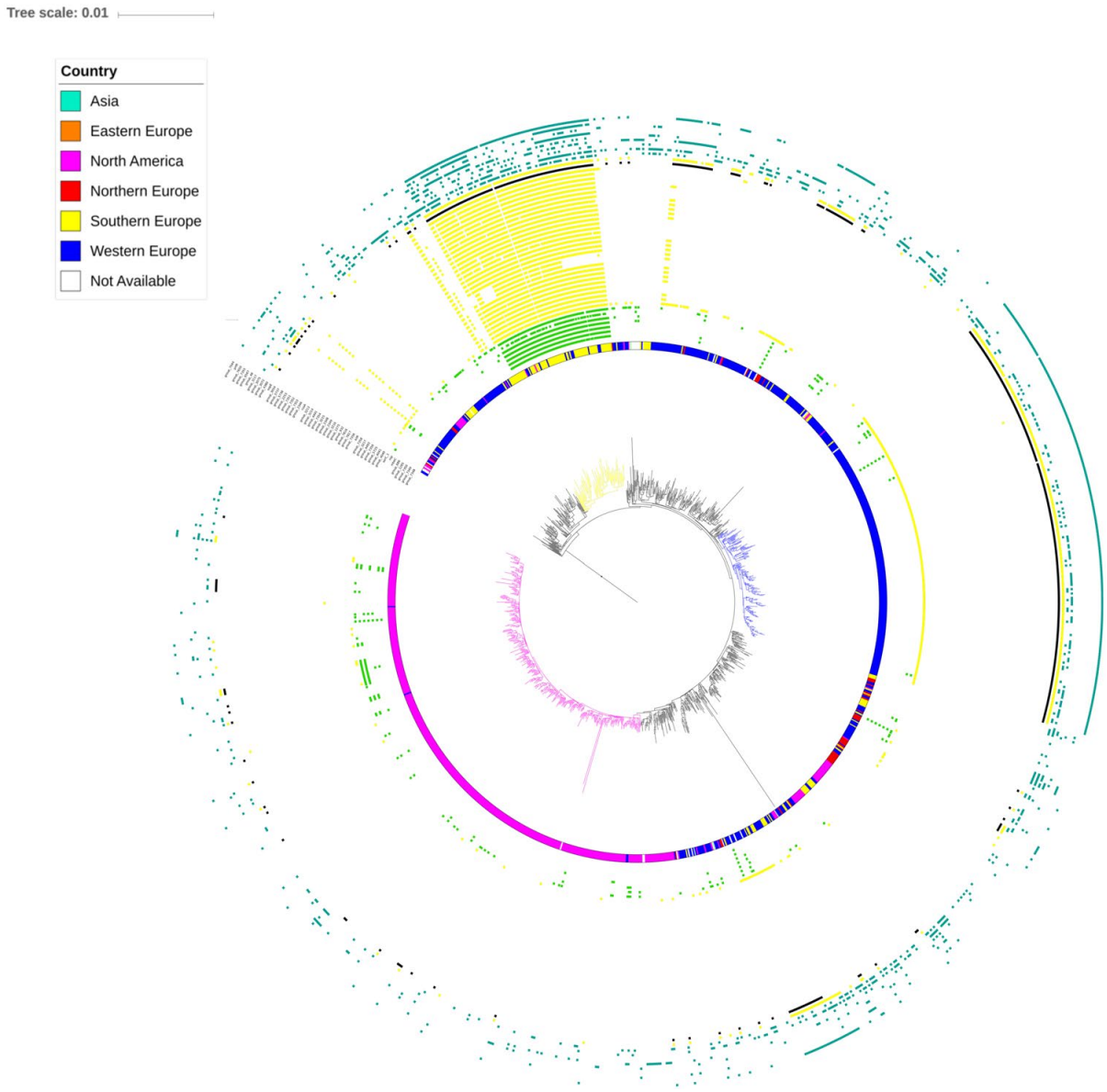

Figure 8: example of core SNPs maximum likelihood tree inferred based on 11,278 core SNPs detected on 1,289 isolates of *Salmonella enterica* ser. Typhimurium. Adapted from Palma et al., (2018).

One critical issue in the analysis of sequencing data is the interpretation of outcomes from a biological (e.g., the presence of a particular gene correlates with the observed phenotype) or epidemiological (e.g., an isolate is part or not of an outbreak) point of view. Bacterial populations within different species and even serotypes tend to be clonal or panmictic, so the biological variation to consider when seeking for epidemiological links should be adapted to the different contexts. Moreover, distinct data analysis pipelines will not measure the same differences. Hence, it is hard to specify a single threshold of SNPs or allele differences to define two isolates as "closely related," and there is still no full consensus within the scientific community on the optimal values to adopt in the different instances (e.g., any type of outbreak or surveillance activity). For example, the U.S. federal agencies defined cutoffs as general recommendations to use in conjunction with complementary information such as species clonality, diversity in the farm-to-fork continuum, the epidemiological question to be addressed as well as the analytical pipeline used. In this perspective, they distinguished three scenarios: (i) isolates that are closely linked, (ii) isolates that are not related, and (iii) isolates that are neither closely related nor unrelated (Brown et al., 2019).

In the first case, the isolates being investigated will generally differ by 0-20 SNPs/alleles. Although they most probably own a recent common ancestor and may share a common source in the production and distribution chain, further information on the product, processing, and supply musts be considered to provide evidence of any link between isolates. When extremely similar isolates are discovered in different sites of a food manufacturing plant, it may indicate a single strain has likely spread across the facility. In such case, additional sampling and research may need to establish the transmission chain. Currently, WGS represents the ultimate tool to elucidate and target the sources of contamination within food facilities and provides a great support to mitigate such problem. Bacterial strains tracing is very challenging because isolates may be related to one another at an unknown earlier phase or location in the production and distribution chain. Thus, a genetic “match” would strongly suggest a common ancestry between isolates, yet this information alone is insufficient for taking regulatory actions (Brown et al., 2019).

Isolates that differ significantly from one another, often by more than 50/100 SNPs/alleles, may not always come from the same source. The presence of several strains on a food production farm or in a facility, on the other hand, may signal unsanitary conditions that must be rectified quickly. Such conditions may underlie so called polyclonal outbreaks, not rare events where numerous pathogenic strains are part of an outbreak linked to a single food source. Finding epidemiological links between relatively close strains is not always obvious. Circumstances in which isolates are not obviously linked but also not genetically unconnected (with 20-50 SNP/allele pairwise differences), are very challenging to interpret. Zoonotic epidemics, where human infections are caused by contact with animals, are striking examples of cases in which an outbreak strain constantly evolves during *in vivo* propagation in the reservoir accumulating enough variation to delineate multiple descendant strains (Brown et al., 2019).

Despite the U.S. methodology, when interpreting sequencing data in surveillance or outbreak investigations, it is generally assumed that isolates significantly similar have a recent common

ancestor, whereas isolates that are less similar are consequently less likely to share a recent common ancestor. This should mean that clinical isolates, as well as clinical and food or environmental isolates, which result as phylogenetically closely related, are thus likely to be epidemiologically or causally associated. Although it is a key premise in molecular epidemiology that biological similarity also reflects epidemiological relatedness, biology does not necessarily correspond with epidemiology 100% of the time (Brown et al., 2019). Because foodborne pathogens have short generation times under standard growth conditions, a small number of genetic changes (e.g., SNPs or allele differences) will accumulate over time, including from the product contamination to the isolation of the clinical specimen or subsequent resampling of a facility or product. As a result, while being part of the same outbreak, it is possible that a minor but detectable difference in the number of SNPs or allele will be identified among clinical, food, animal, and environmental isolates. Thus, despite the genetic variations, these strains will cluster together, separately from unrelated isolates (Brown et al., 2019). Likewise, even if genomic analysis provides strong evidence that isolates are closely related, it does not always imply that a clinical patient was directly infected by a specific food or a specific geographical location where WGS matched isolates were collected. Epidemiological contextualization (e.g., isolates' sources and spatio-temporal distribution) of phylogenetic findings is thus critical to support scientists in the precise identification of the food vehicle, the initial source of contamination as well as the transmission pathways (Jagadeesan et al., 2019). Then, the fixed SNP/allele patterns can be mapped onto a phylogenetic tree and used as biomarkers for specific strains or groups of strains (Brown et al., 2019). In conclusion, because phylogenetic relatedness not always reflects ecology/epidemiology, interpreting sequencing data of strains based on SNP/allele differences requires deep knowledge of the studied pathogen species, its genetic diversity in the farm-to-fork ecosystem and the representativeness of the isolates under examination. This underlines that epidemiological links between isolates cannot be supported by sequencing data alone for food safety surveillance or outbreak investigation (Besser et al., 2018; Jagadeesan et al., 2019; Schürch & van Schaik, 2017).

3.2.3. In silico subtyping of genetic features of concerns (AMR and virulence)

Beyond high-resolution identification of strains, WGS allows to implement *in silico* techniques which provide faster, lower-cost, higher resolution and more robust subtyping data compared to traditional methods (Collineau et al., 2019). One example of this application is for the prediction of AMR phenotypes by detecting *in silico* the presence of AMR genes directly after sequencing the bacterial isolates (Ronholm et al., 2016).

AMR is currently one of the most significant threats to global public health, affecting the worldwide population, animals and the environment. Rise of AMR concerns is driven by the emergence of bacterial strains resistant to one or more antibiotic (AB) therapies, leading to an increased prevalence and severity of infections in clinical settings (Laxminarayan et al., 2013). AMR constitutes a severe health burden worldwide, with an estimated 0.2 million newborn fatalities caused by multi-drug resistance (MDR) pathogen-associated sepsis (Aslam et al., 2021; Prestinaci et al., 2015).

Global AMR mitigation demands a multidisciplinary, multisector, and coordinated strategy to address health hazards at the human-animal-environment interface, which falls within the One Health concept (Robinson et al., 2016). Despite several national and international antibiotic restrictions, the worldwide antibiotic usage grew considerably (between 2000 and 2015), and it has been estimated that consumption will more than quadruple by 2030 (Klein et al., 2018). Furthermore, because of infrastructure initiatives and advances in public access to health care, the low- and middle-income countries (LMICs) will see a significant increase in AB consumption (Auta et al., 2019; Yong Kim et al., 2005).

Inadequate antibiotic usage in animals and humans, polluted settings, and insufficient infection control programs are among the causes of AMR's local and worldwide transmission (Burow & Käsbohrer, 2017; Marti et al., 2014). Two-thirds of global antibiotic usage is employed in livestock farming, although their use in animals as growth promoters has been banned in several countries (Aarestrup et al., 2001). Farm animals are then reservoir of AMR than can be directly or indirectly transmitted to humans through food consumption and close contact with the animal. Nontyphoidal salmonellae, a well-known bacterial pathogen causing gastrointestinal infection worldwide, is one of the leading AMR foodborne pathogens associated with the livestock agriculture sector. AMR *Salmonella* strains are typically disseminated by animal transport and often present in asymptomatic animal carriers which can contaminate poultry and other animal meat products with their feces (Aslam et al., 2021).

The lack of control of antibiotic usage in humans, animals, communities, and their discharge in the connected ecosystems has resulted in the persistence of drug residues or resistance genes in the environment, including soil, water, hospital, industrial, farm waste, and other ecological niches (Huijbers et al., 2015; Marti et al., 2014). For example, wastewater treatment plants, drinking water, and coastal water can be carriers of AMR genes or microorganisms and contribute to their spread, further challenging prevention or management systems (Leonard et al., 2018; Ma et al., 2017).

The food chain represents another critical source of bacterial strains harbouring AMR genes that can be transferred between the environment, animals, plants and humans. AMR bacterial strains can indeed contaminate food during its preparation, and potentially infect humans or operate as a reservoir of ABR genes in the human microbiota (commensals, opportunists, and pathogens) after food ingestion (Bengtsson-Palme, 2017).

The emergence of resistant strains is caused by two major factors: continued antibiotic exposure of susceptible bacteria and their dissemination at the human-animal-environment interface (Munita & Arias, 2016).

A bacterial strain can manifest resistance toward ABs through different mechanisms generally triggered by intrinsic or acquired genetic determinants. Intrinsic resistance is when a bacterial species is naturally resistant to certain ABs without any acquired mutation or gene. Its mechanisms includes reduced permeability of the outer membrane and the natural activity of efflux pumps which reduce concentration of antibiotics inside the cell (Aslam et al., 2021). Besides intrinsic resistance, bacteria may express resistance to ABs after the acquisition of further mutations or genes that may induce

several processes including poor antibiotic penetration, drug efflux, target alteration, and antibiotic inactivation/hydrolysis (Blair et al., 2015).

Currently, the global prevalence of bacterial strains with MDR phenotype linked to acquired resistance determinants is increasing. The synthesis of several enzymes such as extended spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs), and carbapenemases, which are linked to resistance to cephalosporins and carbapenems, by different bacterial species seems at the root of such increase. For instance, the acquisition of AMR gene pools including *bla*TEM, *bla*CTXM, *bla*KPC, *bla*NDM, *bla*VIM, and *mcr*-1 have been linked to the emergence of MDR strains of *E. coli*, *P. aeruginosa*, and others (Aslam et al., 2018).

The second key factor associated with the emergence of resistant mechanisms is pathogen trafficking or spillover across human-animal-environment interfaces (Holmes et al., 2016; Woolhouse & Ward, 2013). Moreover, depending on transmission pathways as well as the degree of antibiotic selective pressure, AMR clones may successfully expand in new niches or new geographical areas and spillover into different host populations once established (Baker et al., 2018; Ward et al., 2016).

Mobile genetic elements (MGEs), and particularly plasmids, often carry AMR genes that can be transferred intra- or inter-species through the mechanism of horizontal gene transfer (HGT) (Chang et al., 2015). These genetic elements can act as a vehicle for resistance dissemination at the human-animal-environment interface, and constitute a major pathway for AMR genes transmission (Perry et al., 2014). For instance, *K. pneumoniae* is recognized as a host of several mobile AMR genes and has played an important role in the global spread of diverse ESBLs and Carbapenemases (Tzouvelekis et al., 2012) using HGT as a mechanism of transmission for large pools of AMR genes (Aslam et al., 2021).

Besides the acquisition of AMR genes, bacterial strains can accumulate point mutations in certain genes that may cause failure of the AB therapy (Schürch & van Schaik, 2017). An example is described by mutations in the *pmr*A/*pmr*B genes, which result in increased resistance to polymyxins in *E. coli* and *Salmonella* (Quesada et al., 2015).

Several intervention strategies should be implemented to reduce the AMR burden following a One Health approach: surveillance and reporting of AMR, tracking transmission dynamics of MDR pathogens at the human-animal-environment interface, awareness, community education as well as policy measures (Aslam et al., 2021). AMR control must be supported and AMR associated causes identified by evidence-based research and monitoring (Aarestrup et al., 2012; Perry & Wright, 2014). Targeted studies are required to gain more knowledge on the different drug resistance mechanisms, predict the occurrence rate of AMR in different ecologies, develop cost-effective and acceptable alternatives to failing treatments, and promote antibiotic stewardship (Aslam et al., 2021; Huijbers et al., 2015; Laxminarayan et al., 2013).

Investigating the molecular epidemiology and genetic relatedness of AMR at the human-animal-environment interface play a fundamental role in lowering the worldwide burden of AMR bacteria, (Aslam et al., 2021). WHO has prioritized the detection of resistant bacteria harbouring AMR genes by employing innovative molecular tools such as real-time PCR, gene capture tools, and WGS, among

others, to develop a criterion for identifying AMR in patients, hospitals, and environments (Lanza et al., 2018; Pärnänen et al., 2019; Tacconelli et al., 2018).

Notably, genomic techniques are particularly useful in discovering AMR mechanisms and reservoirs in diverse One Health contexts as well as investigating innovative therapies to decrease the spread of AMR (Aslam et al., 2021). With the democratisation of genome sequencing in recent years, a promising tool to untangle AMR surveillance is today represented by the estimation AMR rate and prediction of AMR resistance profiles based on the genome of bacterial strains isolated either in clinical settings or farm and food industry and the environment (Aslam et al., 2021). As an example, the introduction of WGS in United States National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) allowed to capture AMR genes from any investigated source (foods and clinical isolates) (Brown et al., 2019). Genome-based AMR prediction is much less time-consuming than phenotyping testing, especially for slow-growing bacteria, and it is then recommended for taking prompt and conscious clinical decisions on AB-treatment strategies (Schürch & van Schaik, 2017). The ability of WGS to assess relatedness between strains from different sources also contribute to the identification of AMR transmission pathways between distinct niches (Griffiths et al., 2017). Moreover, the ability to detect AMR genes in complete or draft genomes has successfully improved with the development of several online software packages and databases. In recent years, the number of tools and databases around AMR has significantly increased, and numerous review papers have been published to synthesize current knowledge on these resources (Hendriksen et al., 2019; Papp & Solymosi, 2022). Although AMR database vary significatively in the amount and type of genes and resistance determinants that may contain, they constitute an unprecedented catalogue of AMR genes and mutations. These can be detected by using a number of bioinformatic tools, some of which allow to identify AMR genes from any sources database, such as the Resistance Gene Identifier (RGI) in the Comprehensive Antimicrobial Resistance Database (CARD) (McArthur et al., 2013), the Antibiotic Resistance Database (ARDB) (Liu & Pop, 2009), and the Resistance Gene Finder (ResFinder) database (Zankari et al., 2012) while others are tuned for the analysis of specific pathogens, like Kleborate for *Klebsiella* spp. and MUBII-TB-DB or Dream TB for *Mycobacterium tuberculosis* (Flandrois et al., 2014; McDermott & Davis, 2021).

Besides AMR, the pathogenicity profile of a given strain can also be estimated through the detection of associated virulence factors on genome sequences. These factors can be summarized in 6 categories, such as i) adherence and colonization factors, ii) Type I to VI secretion systems, iii) immune evasion factors, iv) toxins, v) siderophores for iron absorption and vi) invasion genes (Peterson, 1996). The virulence prediction is challenging and often requires a contextual investigation due to complex regulatory pathways and the impact of regulatory mutations. This highlights that the simple presence or absence of virulence genes may not be sufficient per se to conclude on the virulence or pathogenicity of a bacterial strain. Nevertheless, the *in silico* search of homologs to genes or proteins known to be virulence factors is a common method for predicting virulence profiles from genome data (Uelze et al., 2020). Several databases encompassing virulence genes as well as associated relevant features , such as the Virulence Factor Database (VFDB) (Chen et al., 2016),

Victors (Sayers et al., 2019) and PATRIC (Mao et al., 2015) have been created and made public so that the whole scientific community can easily access and implement them in routine surveillance.
Specific chromosomal genetic elements, such as pathogenicity islands often harbour virulence genes. Interestingly, genes located in these elements typically differ from genes on the rest of the chromosome in terms of nucleotide composition and codon usage bias. Pathogenicity islands are thought to have been acquired by horizontal transfer due to their association with MGEs, tRNA genes, and an accumulation of CRISPR sequences and phage related sequences (Hacker et al., 1997; Ho Sui et al., 2009). The Pathogenicity Island Database (PAIDB) hosts a large collection of pathogenicity islands and other candidate sequences (Yoon et al., 2015). Moreover, the Center for Genomic Epidemiology (CGE) has developed web-based tools to identify pathogenicity islands among *Salmonella* spp. (SPIFinder) as well as acquired virulence genes in several bacterial species (VirulenceFinder) (Joensen et al., 2014; Roer et al., 2016).
In conclusion, WGS technologies have boosted the development of advanced approaches for bacterial genome subtyping which is essential to reach a deeper understanding on global emergence of AMR and virulence. *In silico* subtyping not only helps exploring the dynamics of resistant/virulent strains but also the transmission of associated genes across many sectors including humans, animals, plants, water, and soil (Kraemer et al., 2019; Oniciuc et al., 2018; Wee et al., 2020).

3.3. Computational challenges and bioinformatics

HTS platforms, in addition to lowering costs and analysis time, creates a vast quantity of data, which is revolutionizing the way microbial research and traceability is performed in the food safety field. This implies the use of bioinformatics tools for storing, organizing, and analysing the data (Quijada et al., 2020).
Bioinformatics represent an interdisciplinary field encompassing applied mathematics, statistics, and computer science to analyse and interpret biological data (Carriço et al., 2018). This computational research field require a basic knowledge of programming languages, such as Bash, Perl, Python, and R, and, preferably, familiarity with UNIX-based operating systems such as Linux and MacOS. Generally, Linux has become the UNIX system mainly adopted by bioinformatics scientists, which provides user-friendly interfaces available for many distributions (Ubuntu, Centos, Mint, and so on), all resulting in free license and code. Moreover, the main strength of these platforms is in the usage of the terminal, which needs understanding of the Bash language as a command interpreter.
The execution of computational programs from the terminal is strongly recommended in bioinformatic analyses, since command-line tools result more versatile and scalable than web tools and offer the possibility to wrap multiple tools into pipelines which allow automatization and parallelization of the analyses (Quijada et al., 2020). An increasing number of open-source software for bacterial genomes analysis have been developed in recent years and made available in several platforms and repositories such as GitHub (https://github.com/), Conda/Anaconda (https://anaconda.org/) or SourceForge (https://sourceforge.net/). Besides command-line based tools, web platforms such as RAST, MG-RAST, BIGSdb, EnteroBase or Galaxy (https://usegalaxy.org/)

have been created to integrate command-line programs into a user-friendly interface, automating the computational workflows (Quijada et al., 2020). Public web platforms and databases could be exploited by users for a wide range of bioinformatic usage. Some examples are the Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016), designed by the National Center for Biotechnology Information (NCBI) to annotate bacterial and archaeal genomes, or the BIGSdb.Pasteur.fr, Pubmlst.org and EnteroBase (https://enterobase. warwick.ac.uk/) platforms for bacterial strains typing and nomenclature (MLST, cgMLST and wgMLST). An advantage of web platforms is that they offer a graphical user interface that requires minimal bioinformatics knowledge to apply advanced genomic analysis. By contrast, their pre-formatted system limits user customisation, and often demand access accounts/licenses, upload of data in external databases that may pose confidentiality issues and time to obtain results depending on the job queue in the queried server.

The implementation of bioinformatic analyses could be facilitated by the usage of common infrastructures shared by the same country, as they demand a strong computational power but a basic knowledge for installing and running different programs or software. An example of this is the Cloud Infrastructure for Microbial Bioinformatics (CLIMB, www.climb.ac.uk) which has been developed in 2016 by the United Kingdom to provide cloud-based computation, storage, and analytic tools to the scientific community in clinical microbiology, as well as a variety of bioinformatics training programs (Connor et al., 2016).

Overall, bacterial bioinformatics emerged as a potential scientific topic that is continually evolving to keep pace with advances in bacterial sequencing and with open source bioinformatic tools constantly being developed, maintained and improved (Taboada et al., 2017).

Following sample's collection, DNA extraction and sequencing, the workflow to analyse bacterial genome data relies upon specific bioinformatic software that can be implemented alone or as part of pipelines which combine multiple software to analyse large numbers of samples in parallel (Carriço et al., 2018).

A flaw diagram of the main bioinformatic steps involved in WGS-based (sub)typing and comparative analysis of bacterial strains is provided in Figure 9.

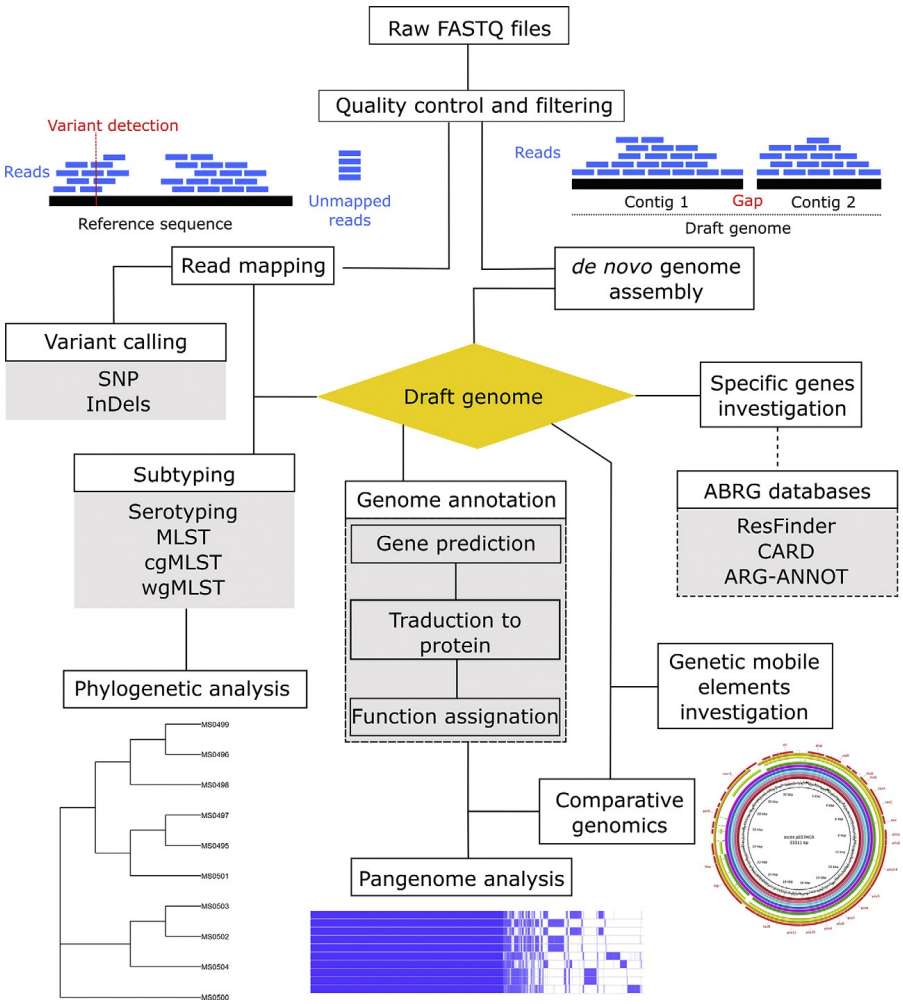

Figure 9: schematic representation of WGS post-sequencing workflow (Quijada et al., 2020).

Sequencing data are produced by most of the modern commercial sequencers (e.g., Illumina) in FASTQ format, which record sequences along with their associated scores called Phred score. Phred scores is a quality measure aimed at quantifying the accuracy of each predicted nucleotide, or base call, included in each read. The first step in a WGS workflow is to quality control and filtering out low quality data from sequenced reads. FASTQC (https://github.com/s-andrews/FastQC) can be used to assess several metrics to define the quality of reads data, including the Phred score of each base call for all reads. Reads are generally cleaned or trimmed to remove low-quality regions and specific regions added during library preparation (index or adapters). A common tool used for this filtering step is TRIMMOMATIC (Bolger et al., 2014). The detection of contamination is another important step to ensure the quality of sequenced reads. These may contain contaminating sequences which belongs to other taxonomic species than the designated strain. The tool Kraken2 (Wood & Salzberg, 2014) can be applied to identify potential contamination by quickly assigning taxonomic origin to each read. Intra-species contamination detection can be performed in raw Illumina data using tools like Confindr (https://github.com/OLC-Bioinformatics/ConFindr) which looks for variants in the conserved core genes catalogued in predefined scheme (ribosomal MLST, cgMLST).

After contaminated sequences filtering, the remaining reads can be used to estimate sequence coverage or depth. The average number of times each nucleotide position in the genome has a read that matches to that position is referred to as coverage. The appropriate coverage values vary depending on the study aims, bacterial species, and the specific downstream analysis. Most FASTQ files in public repositories have coverages ranging from 15 to 500 (Carriço et al., 2018).

After quality control, the most common downstream analysis is genome assembly, where reads are organized into larger sequences named "contigs". Genome assembly can be performed following two approaches: reference-based and *de novo* assembly.

Reference-based assembly consists in mapping reads to a reference sequence, such as a complete or draft genome, plasmid, etc. In reads mapping, multiple reads are aligned to each reference nucleotide and, for each position, the correct base is estimated from the consensus nucleotide obtained from the overlapping reads (Carriço et al., 2018; Ronholm et al., 2016). Other than assembly purpose, read mapping is an accurate approach used in genomic epidemiology studies to assess genetic variation between closely related strain and identify sequence variants (SNPs, insertion or deletion) (Pightling et al., 2014). Examples of popular read mapping software are Bowtie2 (Langmead & Salzberg, 2012) and BWA (Li & Durbin, 2010). Read mappers generate pileup files in the Sequence Alignment Map (SAM) file format, which record every read's position on the chosen reference as well as an alignment score for each read. Other software, such as SAMtools, BCFtools (Li et al., 2009) or Freebayes (Garrison & Marth, 2012) can be used to process SAM files to call the SNPs, insertion or deletion. Snippy represent another commonly used tool to quickly detect SNPs between a haploid reference genome and WGS reads https://github.com/tseemann/snippy. Reference-based techniques, on the other hand, are constrained to the precise selection of the reference genome. When reads are compared to a distantly related reference, only the identical areas are mapped, suggesting that unique sequence data will be removed from the variant report since excluded from the mapping step. Thus, the usability of such methods would depend by the availability of an adequate reference choice, considering that the more distant the selected reference is, the more regions are likely to be omitted from the study (Carriço et al., 2018).

Reference-based methods such as help overcame such issue as they fully rely in the information recorded in the sequence's dataset without mapping against any reference. Reference free approaches are recommended if no relevant reference genome is available for comparison, as they allow to discover the unique genetic content of pools of strains and study genomes' structural variations. Indeed, *de novo* assembly represents the most informative approach when working with new or highly variable bacteria (Loman et al., 2012). *De novo* assembly software employs computationally efficient techniques to detect overlapping reads and expand them into larger contiguous sequences (contigs). This approach typically produces draft genomes, consisting of few to tens or hundreds of contigs, rather than fully assembled ones. The genome assembly is encoded in FASTA format, including for each contig an identifier followed by nucleotide sequences. It is crucial to assess the quality of the assembly to ensure the reliability of following analyses. Multiple metrics, such as the number of contigs, the size of the assembled genome, or the average length of the contigs in the draft genome, can be used to measure the quality of a genome assembly. Another widely used assembly contiguity

metric is the N50 value, which refers to the length of the smallest contig in the set that comprises the fewest (biggest) contigs whose combined length constitutes at least 50% of the assembly (Carriço et al., 2018). A commonly used software to summarise the quality metrics of *de novo* assemblies is QUAST (Gurevich et al., 2013), whereas SPAdes (Prjibelski et al., 2020) is one of the best known genome assembler. Such tools are often wrapped to constitute assembly pipelines, such as INNUca (https://github.com/B-UMMI/INNUca) and shovill (https://github.com/tseemann/shovill), that can be used to generate hundreds or thousands of polished draft genomes from raw reads in a matter of hours. The draft genome represents the starting point to implement further downstream analyses in many studies (taxonomic, functional, genome-wide association).

Genome annotation, referred to as the prediction of the location and the biological role of all genetic features harboured by the bacterium, including protein coding sequences (CDSs), transfer and ribosomal RNA genes (tRNAs, rRNAs) and occasionally operons, CRISPR elements and genomic islands (Carriço et al., 2018) is the most common step after assembling. First, software like Prodigal can be used to obtain nucleotide FASTA files of predicted CDSs and amino acid FASTA files for their translated proteins. The following step consists in aligning the translated proteins against specific databases to return gene taxonomy and function (Quijada et al., 2020). Public databases are available online for genome annotation, such as the National Center for Biotechnology Information (NCBI) prokaryotic genome annotation pipeline (PGAP) (Tatusova et al., 2016) and the RAST Server (Overbeek et al., 2014). A faster and more automated option is the command-line tool PROKKA (Seemann, 2014), which is the gold standard for prokaryotic genome annotation with more than 10’000 citations. This tool can fully annotate a draft genome in about 10 minutes and has a wide range of options, including the possibility to add user-specific databases. The annotation procedure outputs a GenBank file (GBK), which may be investigated for genomic traits of interest and further annotated using free software such as the Artemis genome browser (Rutherford et al., 2000). One of the advantages of annotation is the utility of General Feature Format (GFF) files to reconstruct the entire pangenome repertoire, including both core and accessory genes, within a bacterial dataset. The software Roary (Page et al., 2015) has been developed and largely used by the scientific community to calculate pangenomes across different annotated draft genomes. Combining Roary with statistical tool like Scoary (Brynildsrud et al., 2016), is also possible to perform genome-wide association studies to discover unique genetic markers associated with particular phenotype virulence and host or niche adaptation (Palma et al. 2018).

Beside gene annotation, the screening draft genomes directly against a specific collection of genes, such as those encoding for AMR, virulence or other functions, is commonly performed. ABRicate (https://github.com/tseemann/abricate) and BLASTN (Zhao & Chu, 2014) are common tools used to detect the presence of AMR genes within a bacterial draft genome by querying sequences against curated databases, such as ResFinder (Zankari et al., 2012), CARD (Jia et al., 2017), ARG-ANNOT (Gupta et al., 2014) or NCBI AMRFinderPlus (Feldgarden et al., 2019). Furthermore, the tool PointFinder can be applied to detect point mutations in specific chromosomal AMR genes (Zankari et al., 2017). Such tools can be also used to investigate the presence of virulence genes, using for

example the Virulence Factors Database (VFDB) (Chen et al., 2016) as reference, or any other genes of interest by building customized databases.

Investigating the genetic context and location of detected genes of concerns can be essential to assess their potential mobility (Perry et al., 2014). As MGEs are common shuttles for AMR transmission, plasmids can be reconstructed and typed using a range of software like PlasmidFinder (Carattoli et al., 2014), PlasmidSpades (Antipov et al., 2016), MOB-suite (Robertson & Nash, 2018) and PLACNET (Vielva et al., 2017). In Illumina sequencing technologies, plasmid investigation may be a challenging step because short reads length often fails to cover the highly repetitive sequences seen on mobile elements (Arredondo-Alonso et al., 2017).

Another important step in the analysis of a bacterial pathogen strain draft genome is the definition of its MLST or cgMLST. Three public databases are today the main source of reference MLST/cgMLST schemes and additional (e.g., virulence or AMR) schemes for typing most common pathogens: PubMLST (https://pubmlst.org/), which host a collection of curated databases for over 100 different microbial species and genera, BIGSdb-Pasteur (https://bigsdb.pasteur.fr/), the genomic-based strain taxonomy and nomenclature platform of Institut Pasteur for bacterial pathogens of public health importance, and Enterobase (https://enterobase.warwick.ac.uk/) which hosts MLST/cgMLST schemas for *Salmonella*, *Escherichia*/*Shigella* and *Yersinia* species. In addition to the mentioned public platforms, the command-line software chewBBACA, which allows to create and validate wg/cgMLST schemas and perform allele calling on complete or draft genomes (Silva et al., 2018), is being adopted by several food safety labs for routine surveillance analysis.

A summary list of the bioinformatics software most known for bacterial pathogen strains data analysis, is provided in Figure 10. Bioinformatics analysis has become the rate-limiting phase in the widespread usage of HTS, with fast advances and decrease of DNA extraction and sequencing costs. Since specific technical expertise on how to install and maintain tools and software is required, bioinformaticians represent an essential part of any research group or microbiology unit dealing with HTS data processing. This suggests that non-specialists in bioinformatics should achieve a basic knowledge of the approach and acquire fundamental bioinformatic abilities to productively lead genomic investigations, analyse their results, and improving in their practice (Carriço et al., 2018).

Usage	Software name	Description	URL
Quality measures and read preprocessing	FASTQC	Toolbox for displaying sequence statistics for next-generation sequencing reads	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
	TRIMMOMATIC	Command-line based tool for trimming of short-read paired-end and single-ended data	http://www.usadellab.org/cms/?page=trimmomatic
	FASTX-Toolkit	A collection of command line tools for preprocessing of short-read FASTA/FASTQ files	http://hannonlab.cshl.edu/fastx_toolkit/
	PRINSEQ	Command-line and web-based tool for filtering, reformatting, or trimming genomic and metagenomic sequence data, generates summary statistics in graphical and tabular format	http://prinseq.sourceforge.net/, http://edwards.sdsu.edu/cgibin/prinseq/prinseq.cgi
Contamination detection	Kraken	Taxonomic assignment of reads, useful for metagenomics analysis or detection of contamination in pure culture samples	https://ccb.jhu.edu/software/kraken/
	MIDAS	Taxonomic assignment of reads, useful for metagenomics analysis or detection of contamination in pure culture samples	https://github.com/snayfach/MIDAS
Assembly software and pipelines	Velvet	*De novo* genomic assembler specially designed for short reads	http://github.com/dzerbino/velvet/tree/master
	SPAdes	*De novo* genomic assembler for short reads; it can also provide hybrid assemblies using long-read data together with short-read data	http://cab.spbu.ru/software/spades/
	Canu	*De novo* genomic assembler designed for high-noise single-molecule sequencing such as long reads	http://github.com/marbl/canu
	INNUca	A standardized, fully automated, flexible, portable and pathogen-independent pipeline for bacterial genome assembly and quality control starting from short reads	http://github.com/INNUENDOCON/INNUca
	shovill	A pipeline for bacterial genome assembly which improves SPAdes speed and accuracy	https://github.com/tseemann/shovill
In silico typing	ReMatCh	Software for variant calling based on a read-mapping strategy to selected target sequences; also interacts with European Nucleotide Archive (ENA) repository, easily mining publicly available data	http://github.com/B-UMMI/ReMatCh
	Short Read Sequence Typing for Bacterial Pathogens (SRST2)	It uses short-read data, MLST database and/or database of gene sequences (e.g. resistance genes, virulence genes) and reports the presence of STs and/or reference genes	http://github.com/katholt/srst2
	Microbial InSilico Typer (MIST)	Rapid generation of *in silico* typing data (e.g. MLST, MLVA) from draft bacterial genome assemblies	http://bitbucket.org/peterk87/microbialinsilicotyper
	SISTR	A web- and command line–accessible tool for *Salmonella* typing using draft genome assemblies	http://lfz.corefacility.ca/sistr-app/
	SeqSero	A web-accessible tool for *Salmonella* typing using raw reads or draft genome assemblies	http://www.denglab.info/SeqSero
	RGI-CARD	Curated collection of antimicrobial resistance gene and mutation sequences, bioinformatics models and tools for their detection in bacterial genomes	http://www.card.mcmaster.ca/analyze/rgi
	ResFinder	A web-accessible tool for the detection of acquired antimicrobial resistance genes in bacterial genomes using raw reads or draft genome assemblies	https://cge.cbs.dtu.dk/services/ResFinder/
	VirulenceFinder	A web-accessible tool for the detection of virulence associated genes in *Escherichia coli*, *Listeria* spp., *Staphylococcus aureus*, *Enterococcus* spp. using raw reads or draft genome assemblies	https://cge.cbs.dtu.dk/services/VirulenceFinder/
	MLST1.8	A web-accessible tool for the determination of MLST types from bacterial genomes using publicly available MLST schemas	https://cge.cbs.dtu.dk/services/MLST
	Mlst2.9	Command line–based software which can extract MLST from bacterial genomes using publicly available MLST schemas	https://github.com/tseemann/mlstCFSANSNP
	CFSAN SNP Pipeline	Pipeline for extracting high quality SNV matrices for sequences from closely related pathogens	http://snppipeline.readthedocs.io/en/latest/
	Snippy	A pipeline for rapid identification of haploid variants and construction of phylogeny using core genome SNPs	http://github.com/tseemann/snippy
	SNVPhyl (Single Nucleotide Variant PHYLogenomics)	Pipeline for identifying SNV within a collection of microbial genomes and constructing a phylogenetic tree	http://snvphyl.readthedocs.io/en/latest/
	Lyve-SET	A pipeline for using high-quality SNPs to create a phylogeny, especially for outbreak investigations	https://github.com/lskatz/lyve-SET
Gene-by-gene approaches	BIGSdb	Web-accessible database system designed to store and analyse linked phenotypic and genotypic information, including allele calling engine for gene-by-gene approach; it is the database system for both PubMLST and PasteurMLST	https://github.com/kjolley/BIGSdb, http://pubmlst.org http://bigsdb.pasteur.fr/index.html
	Enterobase	Curated database and online resource for molecular typing of *Salmonella*, *Escherichia coli*, *Yersinia* spp. and Moraxella spp. using gene-by-gene approach	http://enterobase.warwick.ac.uk/
	Genome Profiler	Stand-alone gene-by-gene allele calling algorithm which uses conserved gene neighbourhoods to resolve gene paralogy	http://sourceforge.net/projects/genomeprofiler/
	chewBBACA	A comprehensive and highly efficient stand-alone gene-by-gene allele calling algorithm based on coding DNA sequences, including suite of tools for providing overview of schema performance	https://github.com/B-UMMI/chewBBACA
Gene annotation	Prodigal	Protein-coding gene prediction software tool for bacterial and archaeal genomes	http://github.com/hyattpd/prodigal/wiki
	Prokka	Quick functional annotation of bacterial genomes producing standards-compliant output file	http://github.com/tseemann/prokka
	RAST	Fully automated service for annotating bacterial and archaeal genomes	http://rast.nmpdr.org/
	MicroScope	Comprehensive analytical platform for genome annotation and analysis of bacterial genomes	http://www.genoscope.cns.fr/agc/microscope/home/index.php
	NCBI prokaryotic genome annotation pipeline (PGAP)	Automatic prokaryotic genome annotation pipeline that combines ab initio gene prediction algorithms with homology-based methods	https://www.ncbi.nlm.nih.gov/genome/annotation_prok/
	NCBI Pathogen Detection	An online platform for sharing and comparing data on outbreak strains; currently contains databases for 20 bacterial species, focusing on food-borne pathogens and healthcare-associated infections	https://www.ncbi.nlm.nih.gov/pathogens/
Genome alignments	Harvest	A suite of core genome alignment and visualization tools for quick and high-throughput analysis of intraspecific bacterial genomes	http://harvest.readthedocs.io/en/latest/
	Mauve	Aligner for comparative analysis of full bacterial genomes	http://darlinglab.org/mauve/mauve.html
Homology clustering and Association studies	Roary	High speed stand-alone pan-genome pipeline for bacterial genomes	http://sanger-pathogens.github.io/Roary/
	Scoary	Pan-genome–wide association studies using Roary output	https://github.com/AdmiralenOla/Scoary
	Neptune	Software designed for detecting genomic signatures within bacterial populations	https://github.com/phac-nml/neptune
Phylogenetic inference	RAxML	Sequential and parallel maximum-likelihood phylogeny estimation that operates on nucleotide and protein sequence alignments	https://sco.h-its.org/exelixis/software.html
	FastTree	Compute approximately maximum likelihood phylogenetic trees from large nucleotide or protein multiple sequence alignments	http://www.microbesonline.org/fasttree/
	Gubbins	Compute maximum likelihood from alignment after removing regions containing elevated densities of base substitutions	https://github.com/sangerpathogens/gubbins
	ClonalFrameML	A maximum likelihood implementation of ClonalFrame designed for genomes sequences	https://github.com/xavierdidelot/ClonalFrameML
	PHYLOViZ	Online Web-based tool for phylogenetic inference, visualization, analysis and sharing of sequence-based typing methods that generate allelic profiles and associated epidemiologic data	http://online.phyloviz.net
	PHYLOViZ 2.0	Stand-alone Java software for phylogenetic inference, visualization and analysis of sequence-based typing methods that generate allelic profiles and their associated epidemiologic data	http://www.phyloviz.net/
Visualization tools	Microreact	A web-based tool for genomic epidemiology data visualization and sharing	http://microreact.org
	Phandango	Interactive web-based tool for fast exploration of large-scale population genomics data sets combining output from multiple genomic analysis methods	https://github.com/jameshadfield/phandango
	iTOL	Web-based tool for display, annotation and management of phylogenetic trees	http://itol.embl.de/
	GenGIS 2	Application including 3-D graphical and Python interfaces allowing users to combine digital map data and sequences	http://kiwi.cs.dal.ca/GenGIS/Main_Page
Multipurpose analytical platforms and pipelines	Centre for Genomic Epidemiology Toolbox	A suite of web-based tools and service for pathogen molecular typing, genome assembly, phenotypic prediction (e.g. resistance prediction) and phylogeny construction	http://cge.cbs.dtu.dk/services/
	Integrated Rapid Infectious Disease Analysis (IRIDA) Platform	A Galaxy-based platform for real-time infectious disease outbreak investigation using genomic data including a sequence data management module and workflows, ontology framework (GenEpiO) and data visualization tools	https://irida.corefacility.ca/documentation/downloads/index.html, http://irida.ca/
	Integration genomics in surveillance of food-borne pathogens (INNUENDO) platform	A platform for real-time disease outbreak investigation and surveillance of food-borne pathogens using genomic data including sequence-data management module, assembly modules with QA/QC measures, gene-by-gene analytical pipeline, ontology framework (GenEpiO) and visualization tools	https://github.com/INNUENDOCON/INNUENDO_platform
	Nullarbor	A pipeline for generating public health microbiology reports from sequenced isolates including sequencing specifics, species ID, subtypes and core SNP	http://github.com/tseemann/nullarbor

Figure 10: Description of software, tools and pipelines used in WGS workflow (Carriço et al., 2018).

3.4. Opportunities, challenges and future perspectives of adopting WGS in food safety

Estimating that the world population will be close to reach 9.1 billion people in 2050, agricultural trade is expected to follow this expansion (FAO, 2015). In the last few decades, the global food supply chain has become extremely complex, with ingredients connected to a food item coming from multiple suppliers in different countries (McCullough et al., 2008). This systematic food system suggests that a single local contamination could impact a large portion of the food supply chain, thus reaching more and more people on a global scale (Gharehgozli et al., 2017). This situation could even be worsened by the growing online food trade and direct e-food purchasing which could generate a huge traffic of food items that do not necessarily go under established border control systems (McCullough et al., 2008). As a result, it is necessary to put more efforts on enforcing food safety collaborations among countries by adopt standardized common practices to generate and share genomic data and creating a functional environment that allows effective global management of foodborne disease outbreaks (Fukuda, 2015; Sasaki & Burr, 2000). In the richest countries, WGS is already used as an essential routine tool for identifying and characterizing pathogens in food safety management (Jackson et al., 2016), and many funding campaigns and capacity building initiatives have been carried out in recent years to boost the adoption of genomic-based surveillance of bacteria pathogens at global scale (WHO, 2022). A step forward to genomics data standardization has been recently pursued with the publication of ISO 23418:2022 (https://www.iso.org/standard/75509.html) on Whole Genome Sequencing for typing and genomic characterization of bacteria. This document contains general requirements and guidance for generating and analysing whole genome sequencing (WGS) data of bacteria obtained from the food chain, including:

1- handling of bacterial cultures;
2- axenic genomic DNA isolation;
3- library preparation, sequencing, and assessment of raw DNA sequence read quality and storage;
4- bioinformatics analysis for determining genetic relatedness, genetic content and predicting phenotype, and bioinformatics pipeline validation;
5- metadata capture and sequence repository deposition;
6- validation of the end-to-end WGS workflow (fit for purpose for intended application).

This document is applicable to bacteria isolated from:

- products intended for human consumption;
- products intended for animal feed;
- environmental samples from food and feed handling and production areas;
- samples from the primary production stage.

The advantage of using WGS for epidemiological investigations and surveillance of bacterial foodborne pathogens lies in the fact that it constitutes a one-stop solution to quickly identify emerging health threats by investigating genotypes, virulence, AMR, and other relevant features with a single method (Nadon et al., 2017; Taboada et al., 2017). Moreover, WGS allows to share high-resolution,

standardized and comparable data, useful to identify genetic variants and investigate the effects of expression and regulation important genes, across labs and institutions (Gilad et al., 2009). Several countries have adopted WGS data-sharing and storing systems, and some key databases and platforms are today routinely use for this purpose, such as the US FDA GenomeTrakr (Allard et al., 2016), a subset database of the Sequence Read Archive at the NCBI (https://www.ncbi.nlm.nih.gov/sra), the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home), and the deoxyribonucleic acid (DNA) Data Bank of Japan (https://www.ddbj.nig.ac.jp/index-e.html).

Following the progresses of WGS from a research tool to a practical instrument for food safety management, an large number of bioinformatics software have been developed to analyse foodborne pathogen sequence data (Langmead & Nellore, 2018). These tools allow to generate and collect huge volume of high-quality data in a short period of time making this approach far more efficient and precise than conventional molecular techniques (Daetwyler et al., 2014). Consequently, WGS has successfully contributed to foodborne outbreak investigations in countries where this technology has been timely adopted, demonstrating its key role in food safety (Wang et al., 2016).

Whilst WGS has widely revolutionized molecular typing of bacterial pathogens, various scientific gaps and technological challenges still need to be addressed to improve WGS data interpretation and enable its broader use in food safety management for the food industry. Future advancements of sequencing platforms and yields will be strongly connected to the development of massive amounts of data, thus requiring dedicated bioinformatics capabilities for their analyses and interpretation. Lack of advanced computational resources and skills in most laboratories is a major obstacle to WGS implementation worldwide. This underlines the importance of establishing dedicated training programmes to form personnel both at the regulatory and food sector levels to deal with WGS data. To bridge this gap, non-bioinformaticians can take advantage of the user-friendly bioinformatic solutions today available (e.g., the PathogenWatch platform) to efficiently conduct WGS data analysis (De Filippis et al., 2018; Kwong et al., 2015; Moran-Gilad, 2017). Although such solutions are extremely useful to the scientific community, command-line tools and pipelines are still needed to enable scalability, automation and parallelization of WGS big data analysis. Open access and sharing of software, pipelines and genomic data into public repositories which are maintained and updated by international community is today an asset for the adoption of WGS at global scale. Researchers may indeed profit from the unparallel information and tools publicly available while contributing to the community's knowledge by sharing their own data and tools. The always increasing volume of genomic data combined with advanced approaches for their analysis will continue to advance microbial populations studies and understanding of their evolution, epidemiology, survival capacity, stress adaptability, and mechanisms for the spread of virulence and/or antibiotic resistance determinants (Hoelzer et al., 2018).

Considering all its advantages, WGS is currently the official method of food safety management systems in developed countries, yet its adoption by government systems in developing countries as official food safety management tool is pending (Pekdemir, 2018). Main obstacles are the overall cost to sustain the implementation of WGS and the slow delivery of sequencing kits and laboratory reagents. However, investing on WGS can help in the long term to reduce the cost of identifying

foodborne pathogens, of each sequencing run and of the overall workflow, which is still high since the technology requires necessary infrastructure and operational equipment/personnel (FAO, 2016). Another challenge is the lack of efficient diagnostic services for reporting illnesses in less resourced countries, which results in a loss of sensitivity and specificity of data collected for disease surveillance. Before using WGS, governments should set up a systematic process to collect and collate data on bacterial pathogen isolates and associated information from clinical as well as food/environment samples (EFSA, 2008; FAO, 2016). Therefore, effective incorporation of globally harmonized rules and regulations, along with standard practices that ensure interoperability of genomic data from bacterial pathogens (Timme et al., 2020) is worthy to assist developing nations in dealing with all these issues.

To sum up, infrastructure and technical capacities, including laboratory conditions and bioinformatic skills, continue to hinder the use of WGS in less resourced countries (Ahmed et al., 2015). This results in a global concern because the core nature of WGS largely lies in data sharing for real-time access to worldwide data (Apruzzese et al., 2019), while partial collection of data from limited geographical areas limits the benefit WGS can provide to global surveillance of foodborne pathogens (Kaye et al., 2009). Investments in training and infrastructure, tailored scientific collaborations and robust legislative frameworks would help overcome challenges and fulfil the knowledge gaps in employing WGS as an integrated solution to national food control systems also in developing countries, and ultimately achieve a successful transition from conventional methods to WGS in public health microbiology (Hoelzer et al., 2018).

Food safety issues frequently result in large-scale financial losses as a consequence of product recalls, disposals, and penalties, as well as significant harm to the entire reputation of food manufacturers, corporations, and nations, leading to loss of customer trust (Hussain & Dawson, 2013). A routine application of WGS could help minimizing unnecessary food recalls by reducing source-attribution errors and, eventually, food waste. Few companies are currently using WGS to monitor bacterial strains and determine contaminated niche inside their production lines, thus preventing finished products from contamination. The use of WGS by food industry may have the greatest potential impact on food safety and public health by drastically lowering the number of contaminated items entering the market. It is expected that when more definitive WGS information on the diversity of bacterial features linked to adaptability, survival, competitive ability, and metabolic preferences becomes available, the application of WGS will broaden the focus from infections to food degradation and shelf life. Likewise, a deepest knowledge on bacterial genotypes and genes that affect public health or food product deterioration can assist the industry in producing safer foods with longer shelf life and higher quality (Brown et al., 2019).

In conclusion, WGS and its wide applications is today a tangible solution to improve bacterial foodborne outbreak and surveillance investigations as well as source attribution (i.e., the capacity to correlate sporadic disease to particular foods or food categories). The usage of WGS to investigate specific or concerning genes associated with virulence, pathogenicity, survival, adaptability, antimicrobial and biocide resistance, food quality and spoilage, and how these genes spread within

microbial communities will help to improve not only the preventive and control aspects of food safety and public health, but also the quality and shelf life of foods (Brown et al., 2019).

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Aims of the work

Artisanal food productions are intrinsic elements of regional and local cultures. Most of these productions undergone to fermentation processes by taking advantage from the autochthonous microbial communities associated with typical raw ingredients and the production environment, being responsible of the final products richness of taste. The support for ethical and ecological issues, along with attractive organoleptic features boost consumer's acceptability and drive their increasing popularity all around the world (Cirne et al., 2019).

Europe is known for its abundance of traditional food products, with the Mediterranean area embracing a rich traditional food identity due to a higher market of small companies, which foster a high degree of biodiversity on artisanal food items (Jordana, 2000). Among the artisanal Mediterranean food chain, traditional dairy and meat fermented products embody a robust food culture that quickly evolved into a fast-growing niche market (Barbieri et al., 2021; Hinrichs, 2004). Nevertheless, lack in full automation and standardization of processing conditions as well as possible heterogeneity of raw materials pose several challenges for small-scale artisanal producers in monitoring environmental parameters and ensuring the microbiological safety of their products, especially ready-to-eat food. In this context, it is crucial to investigate and monitor microbial hazards through the artisanal food supply chain, to protect public health while verifying compliance with current legislative standards (Regulation (EC) No. 2073/2005).

WGS has risen as a universal and powerful tool revolutionizing the molecular landscape in food safety investigations. Compared to traditional methods, it allows to decipher strain diversity at single-nucleotide level of the core- or even whole genome scale, providing ultimate resolution to infer pathogen transmission dynamics along the food chain (Ronholm et al., 2016). In addition to provide high-resolution typing, WGS enables to elucidate genetic key traits such as pathogenicity and virulence, adaptation and survival, resistance to biocides, metals, antimicrobial drugs, and the plasticity of genomes (Brown et al., 2019). Several specialized software for bacterial genome analysis have been developed and constantly improved in the last years, making bioinformatics essential for scientists to extract relevant and meaningful information from the wide array of WGS data (Carriço et al., 2018). Democratization of WGS and availability of analytical tools with unprecedent resolution power, made of bacterial genomics the method of choice for national and international surveillance of foodborne pathogens along the food chain, including the artisanal one (Brown et al., 2019).

The present PhD thesis aimed at assessing the prevalence and growth of foodborne pathogens and other microbial populations, inferring pathogens transmission dynamics, and exploring genetic traits of concern (AMR and virulence) along the food chain of meat and dairy artisanal food productions typical of the Mediterranean countries. These goals were pursued through three distinct studies, combining conventional typing techniques with WGS and advanced bioinformatics approaches, to gain a broader view of the concrete microbial hazards threatening productions.

The first study aimed at evaluating the microbiological quality of two Italian artisanal fermented productions of dairy and meat origin (soft cheese from pasteurized cow milk and salami obtained from pork meat) and investigating influence of seasonality in terms of microbial growth and presence

of biological hazards. To address this goal, samples of raw materials, semi-finished and finished products as well as processing environments were taken from 6 batches along one year, and further tested for the occurrence of main biological hazards (*L. monocytogenes*, *S. aureus*, *Salmonella spp*. and VTEC) as well as enumeration of microbial families used as food quality indicators (Total bacterial count (TBC), lactic acid bacteria (LAB) and *Enterobacteriaceae* (ENT)) and physicochemical parameters (pH and water activity). Additionally, members of the Enterobacteriaceae family were isolated and identified at species level.

The goal of the second study was to assess the occurrence of *Listeria monocytogenes*, *Salmonella enterica* and *S. aureus* strains and investigate their AMR and virulence key traits within a broader range of artisanal fermented meat and dairy productions typically consumed in the Mediterranean area, such as soft cheese and fermented sausages or salami from Portugal, Spain, Italy and Morocco. These objectives have been carried out by applying a genome-based 7-loci MLST and SNP calling to assess strains phylogenetic relationships and screening genomes for antimicrobial resistance or virulence genes detection against public databases (Resfinder and VFDB).

The third study was set up in the light of results obtained from the Italian cheese and salami artisanal producers indicating the presence of *Klebsiella* spp. strains in both facilities, and of increasing reports on *K. pneumoniae* strains with AMR and virulence traits from food and farm animals which raise concerns about their potential role as foodborne pathogens. The study has been then focused on investigating *Klebsiella* spp. strains (broadly comprising *K. pneumoniae*, *K. oxytoca* and *K. planticola* species complexes) from a large collection of salami and cheese samples covering a sampling period of six commercial batches, to gather evidence on the public health risk that artisanal RTE foods may pose as vehicles of *Klebsiella*. The main goals of the study were to (i) assess the occurrence of *Klebsiella* spp. strains through the selected food productions and performed genomic sequencing to (ii) improve their taxonomic characterization and (iii) describe the circulating genotypes and their genetic features of proven clinical importance (i.e., antimicrobial resistance and virulence). Finally (iv) *Klebsiella* spp. sequences were compared with public databases to evaluate the genetic relatedness between newly sequenced food isolates and public ones belonging from other ecological niches.

Results expected from this work will help to expand current knowledges on microbial hazards associated to the artisanal food chain of animal origin and provide further insights to support enhanced surveillance of bacterial pathogen strains.

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Study 1

Investigation on the microbiological hazards in artisanal cheese and salami produced in Northern Italy and its production environment in different seasonal periods

Abstract

The present study aimed at assessing the occurrence of microbiological hazards (*Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella spp*. and *Escherichia coli* VTEC) in artisanal soft cheese and salami produced in Northern Italy in two batches collected along one year of sampling. Furthermore, enumerations of total bacterial count (TBC), lactic acid bacteria (LAB) and *Enterobacteriaceae* were performed as well as monitoring of water activity and pH. Samples were collected from both cheese and salami artisanal facilities from processing environments, raw materials, semi-finished and finished products.

In cheese, a seasonality influence was pointed out by the higher load of TBC in winter, which significantly increased at the end of the cheese storage together with LAB. Moreover, the winter batch showed higher values of pH in raw materials and at the end of storage. Likewise, an overall increase of TBC and LAB was highlighted for salami during the ripening period, showing higher growth in the summer production. Meanwhile, a drop was exhibited in enterobacterial load mainly because of pH and lower water activity values, suggesting the ripening phase leaded by indigen autochthonous strains effective in reducing the microbial burden in salami. Concerning pathogens detection, few stains of *S. aureus* were confirmed in the winter season from the stored cheese, whereas among the *Enterobacteriaceae* family *Klebsiella* spp. strains (*Klebsiella pneumoniae* and *Klebsiella oxytoca)* were detected in both artisanal productions over summer and winter seasons, disseminating through the environment and along salami and cheese-making processes.

Overall, seasonality in artisanal cheese and salami is likely to affect microbial growth, thus focusing the attention on monitoring microbial growth and enhancing disinfection procedures to avoid cross-contamination events. Nevertheless, further studies are required to investigate the burden and public health implications of *K. pneumoniae* and *K. oxytoca* isolates along the artisanal food chain.

Introduction

The consumer demand of artisanal foods has grown in popularity in recent years. Artisanal foods have come to be perceived as more genuine and nutritious compared to their industrial counterparts, showing small scale products with high quality and low availability often generating a positive image of health and ethicality (Almli et al., 2011; Roccato et al., 2017).

Among the European artisanal foods landscape, traditional dairy products (e.g., cheese) and fermented meat represent a unique cultural heritage, especially in Mediterranean countries, where several local productions are widely consumed and appreciated (Bassi et al., 2022; Salameh et al., 2016). On the other side, standardization of productive parameters and automation are more challenging in artisanal small-scale facilities. A less standardized process suggest a potential variability in the product's intrinsic proprieties, which often depends on the type and amount of bacteria in the food. Microorganisms can persist in the production environment on both non-food contact and food contact surfaces and raw materials. A lack of hygiene procedures and adequate sanitary training of the production staff could lead to cross contaminations from the environment and/or from food operators to the product in all the production processing steps (Halagarda & Wójciak, 2022; Omer et al., 2018; Roccato et al., 2017; Thévenot et al., 2005).

From a food safety perspective, soft cheeses can possibly act as a vehicle of transmission to humans of biological hazards, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella spp*. and verotoxigenic *Escherichia coli* (VTEC) (Choi et al., 2016; Holck et al., 2017; Moore, 2004).

The spread of these biological hazards in small scale artisanal facilities with less standardized productions draw an increasingly attention in monitoring the microbial quality of both food matrices and corresponding environmental sites to minimise safety risks for consumers.

In this framework, this study aimed to evaluate the burden of microbiological hazards in two Italian artisanal food fermented productions of dairy and meat origin (soft cheese and salami) while capturing potential differences between winter and summer productions. To achieve this, samples of raw materials, semi-finished and finished products as well as processing environments were taken for two batches of each production, and further tested for the investigation of the occurrence of main biological hazards (*L. monocytogenes*, *S. aureus*, *Salmonella spp*. and VTEC), enumeration of Total Bacterial count, Lactic acid Bacteria and *Enterobacteriaceae*) as well as physicochemical parameters (pH and water activity).

Materials and methods

Experimental design

In the present study, two artisanal food productions of dairy and meat origin were investigated.

The dairy production was linked to a traditional soft cheese made from pasteurized cow's milk added by starter cultures (e.g., *Streptococcus thermophilus*), calf rennet, and enzymes, which sensorial appearance is shapeless and grainy with a delicate and sweet taste. Whereas, the meat production was represented by an artisanal salami made by a mixture of ground swine meat from an autochthonous breed added with spices (garlic and pepper) but lacking industrial preservatives (e.g., nitrates and

nitrites), stuffed in a natural casing. Both productions are Ready-to-eat, which means that they are intended by the producer for direct consumption, without the need for cooking or other processing. Cheese and salami were manufactured in two distinct artisanal small-scale facilities located in Northern Italy. For each artisanal foods, two batches were investigated, respectively produced in different seasons, such as summer (July 2020 for both cheese and salami) and winter (November 2020 for salami and January 2021 for cheese). Samples (n=5 replicates) were collected from raw materials, semi-finished and finished products as well as processing environments (Table 1) according to cheese and salami processing flowcharts (Supplementary Figure 1). Environmental samples were collected by swabbing a 100 cm^2 area with a sterile cotton swab (Copan Italia, Brescia, Italy) that has been moistened in 10 mL of saline solution (0.9% NaCl), before cleaning and disinfection procedures. Notably, the shelf-life period was investigated for cheese at three different temperatures: 2°C (recommended), 8 °C (abuse), 2 °C for 5 days and the remaining 10 days at 8 °C (dynamic) as for salami the expiration date was not declared according to EU regulation (European Union, 2018). In total 250 and 140 samples were collected considering the two batches from artisanal cheese and salami production plant respectively.

Microbiological enumeration, physicochemical properties and biological hazards identification

All samples were tested for the enumeration of Total Bacterial count (TBC) (ISO, 2013), whereas Lactic acid Bacteria (LAB) (ISO, 1998) and *Enterobacteriaceae* (ISO, 2017) were enumerated in raw materials and semi-finished and finished products. Physicochemical analyses of pH (ISO, 1999) and water activity (ISO, 2004a) were performed on raw materials, semi-finished and final products.

Pathogen's detection was carried out in all samples for *Listeria monocytogenes* (ISO, 2017c), *Salmonella spp*. (ISO, 2017a), *Staphylococcus aureus* (ISO, 2004b) and *E. coli* O157 (ISO, 2001). Moreover, for the isolation and identification of bacteria belonging to the *Enterobacteriaceae* family, one aliquot of 25g from samples of raw materials as well as semi-finished and final products along with swabs picked up from the processing environment were enriched in buffered peptone water (Thermo Scientific™) at 37 °C overnight. BPW pre-enriched cultures were then streaked with a 10-µL loopful on MacConkey agar (Thermo Scientific™) and incubated again for 24 h at 37 °C. After plates isolation on selective culture media, colonies were selected based on typical morphology and confirmed by biochemical test (RapID™ ONE System and RapID™ STAPH PLUS System, Thermo Scientific™) and PCR (Chander et al., 2011; Perelle et al., 2004; Saraiva et al., 2018; Wesley et al., 2002).

Statistical analyses

Data obtained were submitted to analysis of variance (ANOVA test) followed by a Scheffé test for post-hoc comparative analysis to detect any significant differences ($p < 0.05$) between batches, days and temperatures of storage (cheese) as well as batches and days of ripening (salami).

Artisanal RTE food production	Sample origin	Description	Number (n)
Salami	Environmental	Wall inside stuffing, drying and ripening area	30
		Manhole inside stuffing, drying and ripening area	30
		Processing surfaces inside stuffing area	10
		Filler stuffer machine located within the stuffing area	10
	Raw materials	Minced meat mixture managed within the stuffing area	10
	Semi-finished products	Salami stored for 1 week inside drying area	10
		Salami stored for 3 weeks inside ripening area	10
		Salami stored for 10 weeks inside ripening area	10
		Salami stored for 18 weeks inside ripening area	10
	Final products	Ripened salami (stored for 28 weeks inside ripening area)	10
Cheese	Environmental	Wall inside warm, maturation and packaging area	30
		Manhole inside warm, maturation and packaging area	30
		Gloves of operators working in the packaging area	10
	Raw materials	Raw and pasteurized milk, calf rennet	30
	Semi-finished products	Raw cheese after warm room and maturation room storage	20
	Final products	Cheese packed on the same day ("day 0") of production along with stored cheese at day 4, 8, 11 and 15 at 2°C, 8°C and 2°C for 5 days followed by 8°C for 10 days.	130

Table 1: Details and number of samples collected *in-situ* at both artisanal food processing facilities (salami and cheese), encompassing for each production raw materials, semi-finished and final product as well as processing environment. The number of collected samples is cumulative to all areas mentioned in the description.

Results

Microbiological quality and physicochemical properties

A total of 250 and 140 samples were overall collected for cheese and salami artisanal productions respectively, in summer and winter seasons.

Table 2 shows results and temporal trends related to enumerations and physicochemical measures in cheese. In both batches, a drop of TBC was noticed after milk pasteurization (summer from 6.28 to 3.52 and winter from 6.56 to 2.36 $\log_{10}$ CFU/ml). As expected, after the addition of starter cultures and rennet to milk as well as the further cheesemaking phases, their load turned to increase until the end of storage. Notably, this growth was higher for the winter batch compared to summer, reaching significantly higher values over the 11^{th} and 15^{th} day at 8°C and 2/8°C. No significant differences were captured between batches when comparing the environmental sites, with the highest values (7.35 $\log_{10}$ CFU/g for summer; 6.88 $\log_{10}$ CFU/g for winter) linked to manhole located in the warm room. Similarly, the load of LAB was likely to increase in both batches during the shelf-life storage. In the winter batch, this increase ranged from 3.52 to 3.97 $\log_{10}$ CFU/g considering all tested temperatures, thus showing a statistically significant increase at the end of the storage compared to cheese produced in summer (from 1.47 to 2.75 $\log_{10}$ CFU/g). All samples from the cheese production line showed

values of *Enterobacteriaceae* under the detection limit (<10 $\log_{10}$ CFU/g) in both batches. Concerning physicochemical results, pH slightly decreased after the inclusion of starter cultures and along cheesemaking, although significant higher values were registered among the winter production in raw material (calf rennet), semi-finished (cheese at the end of maturation) and final products (cheese after being packed and at the last day of storage). In particular in the winter batch at the end of storage a pH higher than 5.3 was registered. pH above this value have been described as associated to *Staphylococcus* spp. survival and growth in cheese. No significant differences arose for water activity when comparing seasonality.

Moving to salami, enumerations and physicochemical results are reported in Table 3. For both batches LAB and TBC growth boosted from raw meat mixture up to the 10th week of ripening, highlighting an average increase of 5.56 and 4.68 $\log_{10}$ CFU/g respectively. Nevertheless, over the remaining ripening process until the final products, LAB and TBC growth decreased down to values observed across the first weeks. Statistically significant influence of seasonality was observed during ripening with a higher load of TBC and LAB in the summer batch in comparison to winter. Notably, in both batches physicochemical values of pH slightly increased thorough ripening, although the final products reported values ≤6.26 and ≤6.02 for summer and winter respectively, whereas both water activity decreased over time to 0.88 as a consequence of drying. Regarding the processing environment, an increase of TBC was observed through the salami production line from mixture to ripening areas, with the lowest load associated to the filler stuffer machine within the mixture room (2.9 $\log_{10}$ CFU/cm^2 in average) and the highest to the ripening environment (6.93 $\log_{10}$ CFU/cm^2 in average). Finally, in both batches *Enterobacteriaceae* decreased from approximately 4 $\log_{10}$ CFU/g to values close to the detection limit.

Detection of biological hazards

Concerning results on tested biological hazards (Figure 1, Table 4) *L. monocytogenes, Salmonella* spp. and VTEC were never detected among both productions. Notably, four *S. aureus* isolates were collected from cheese of the winter batch during storage as well as two *S. aureus* in salami, from the raw meat mixture of the summer batch (n=1) and in the semi-finished salami from the drying room in winter (n=1). Moreover, during the identification at species level of isolates belonging to *Enterobacteriaceae,* colonies of *Klebsiella pneumoniae* and *Klebsiella oxytoca* were identified by biochemical tests (RapID™ ONE System) and further confirmed with PCR (Chander et al., 2011). Overall, 13 and 14 *Klebsiella* spp. were collected from cheese and salami respectively. *K. oxytoca* was mostly found in cheese (85%; 11/13 samples) whereas *K. pneumoniae* in salami (93%; 13/14 samples), thereby highlighting a strong association between *Klebsiella* species and tested food productions. In cheese, *K. oxytoca* was recovered in both summer (n=2) and winter (n=9) from the processing environment, semi-finished and final products. Likewise, *K. pneumoniae* was retrieved in summer (n=5) and winter (n=8), from all samples' origin (raw materials, semi-finished salami and environment) except final products. In contrast to cheese, no microbiological hazards were found in

final products suggesting the 6-month ripening associated to the low water activity, as an effective control measure.

Lactic acid bacteria (LAB)		
Sample	**Summer batch**	**Winter batch**
Raw materials and semi-finished products		
Milk post pasteurisation	1.00±0.22	<1.00±0.00
Cheese at the end of the storage in warm room	2.50±0.82	<1.00±0.00
Cheese at the end of maturation	1.67±0.19	1.35±0.41
Final products		
Cheese after packaging	1.64±0.27	1.34±0.60
Cheese packed day 1 at °2C	2.02±0.48	1.28±0.43
Cheese packed day 1 at °8C	1.35±0.77	2.07±1.22
Cheese packed day 1 at 2/8°C	1.75±0.23	1.26±0.40
Cheese packed day 4 at °2C	1.95±0.14	1.33±0.76
Cheese packed day 4 at °8C	1.79±0.35	1.14±0.72
Cheese packed day 4 at 2/8°C	1.80±0.17	1.24±0.69
Cheese packed day 8 at °2C	2.19±0.57	1.25±0.71
Cheese packed day 8 at °8C	3.62±0.27	3.70±0.19
Cheese packed day 8 at 2/8°C	1.91±0.42	2.13±0.56
Cheese packed day 11 at °2C	2.17±0.41	1.27±0.76
Cheese packed day 11 at °8C	3.06±0.46[a]	4.76±0.18[b]
Cheese packed day 11 at 2/8°C	2.47±0.39[a]	3.94±0.58[b]
Cheese packed day 15 at °2C	2.11±0.23[a]	4.86±0.03[b]
Cheese packed day 15 at °8C	4.31±0.46	5.31±0.18
Cheese packed day 15 at 2/8°C	2.40±0.61[a]	4.99±0.10[b]
Total bacteria count (TBC)		
Raw materials and semi-finished products		
Milk before pasteurisation	6.28±0.22	6.56±0.11
Milk post pasteurisation	3.52±0.13	2.36±0.30
Calf rennet	0.90±0.83	2.76±0.56
Cheese at the end of the storage in warm room	5.03±0.09	5.27±0.07
Cheese at the end of maturation	5.59±0.09	5.87±0.05
Environmental samples		
Walls in warm room	4.32±0.46	2.86±0.43
Manhole in warm room	7.35±0.28	6.88±0.44
Walls in maturation room	2.52±1.90	3.75±0.15
Manhole in maturation room	4.70±1.55	5.40±0.51
Operators gloves in packaging room	2.61±0.45	2.69±0.75
Manhole in packaging room	5.24±0.27	4.64±0.68
Final products		
Cheese after packaging	5.63±0.06	5.90±0.16
Cheese packed day 1 at °2C	5.48±0.09	6.00±0.12
Cheese packed day 1 at °8C	5.50±0.04	5.87±0.09
Cheese packed day 1 at 2/8°C	5.48±0.06	5.96±0.09
Cheese packed day 4 at °2C	5.50±0.12	5.93±0.12

Cheese packed day 4 at °8C	5.59±0.05	5.92±0.03
Cheese packed day 4 at 2/8°C	5.55±0.09	5.92±0.07
Cheese packed day 8 at °2C	5.46±0.11	6.21±0.06
Cheese packed day 8 at °8C	5.51±0.07	6.48±0.43
Cheese packed day 8 at 2/8°C	5.52±0.08	6.14±0.08
Cheese packed day 11 at °2C	5.53±0.08	6.22±0.05
Cheese packed day 11 at °8C	5.61±0.13	7.33±0.10
Cheese packed day 11 at 2/8°C	5.64±0.22[a]	7.71±0.17[b]
Cheese packed day 15 at °2C	5.59±0.04	6.84±0.34
Cheese packed day 15 at °8C	5.72±0.07[a]	8.03±0.43[b]
Cheese packed day 15 at 2/8°C	5.64±0.03[a]	7.71±0.61[b]
Enterobacteriaceae		
Raw materials and semi-finished products		
Milk before pasteurisation	<1.00±0.00	<1.00±0.00
Milk post pasteurisation	<1.00±0.00	<1.00±0.00
Calf rennet	<1.00±0.00	<1.00±0.00
Cheese at the end of the storage in warm room	<1.00±0.00	<1.00±0.00
Cheese at the end of maturation	<1.00±0.00	<1.00±0.00
Final products		
Cheese after packaging	<1.00±0.00	<1.00±0.00
Cheese packed day 1 at °2C	<1.00±0.00	<1.00±0.00
Cheese packed day 1 at °8C	<1.00±0.00	<1.00±0.00
Cheese packed day 1 at 2/8°C	<1.00±0.00	<1.00±0.00
Cheese packed day 4 at °2C	<1.00±0.00	<1.00±0.00
Cheese packed day 4 at °8C	<1.00±0.00	<1.00±0.00
Cheese packed day 4 at 2/8°C	<1.00±0.00	<1.00±0.00
Cheese packed day 8 at °2C	<1.00±0.00	<1.00±0.00
Cheese packed day 8 at °8C	<1.00±0.00	<1.00±0.00
Cheese packed day 8 at 2/8°C	<1.00±0.00	<1.00±0.00
Cheese packed day 11 at °2C	<1.00±0.00	<1.00±0.00
Cheese packed day 11 at °8C	<1.00±0.00	<1.00±0.00
Cheese packed day 11 at 2/8°C	<1.00±0.00	<1.00±0.00
Cheese packed day 15 at °2C	<1.00±0.00	<1.00±0.00
Cheese packed day 15 at °8C	<1.00±0.00	<1.00±0.00
Cheese packed day 15 at 2/8°C	<1.00±0.00	<1.00±0.00
pH		
Raw materials and semi-finished products		
Milk before pasteurisation	6.78±0.01	6.76±0.01
Milk post pasteurisation	6.71±0.03	6.72±0.02
Calf rennet	5.09±0.00[a]	5.30±0.04[b]
Cheese at the end of the storage in warm room	5.87±0.04	5.81±0.04
Cheese at the end of maturation	5.27±0.03[a]	5.54±0.05[b]
Final products		
Cheese after packaging	5.24±0.02[a]	5.47±0.03[b]
Cheese packed day 1 at °2C	5.36±0.02	5.32±0.08
Cheese packed day 1 at °8C	5.35±0.01	5.36±0.02
Cheese packed day 1 at 2/8°C	5.37±0.02	5.26±0.03

Cheese packed day 4 at °2C	5.37±0.04	5.36±0.03
Cheese packed day 4 at °8C	5.29±0.02	5.31±0.02
Cheese packed day 4 at 2/8°C	5.28±0.02	5.36±0.02
Cheese packed day 8 at °2C	5.41±0.13[b]	5.23±0.03[a]
Cheese packed day 8 at °8C	5.24±0.01	5.25±0.02
Cheese packed day 8 at 2/8°C	5.28±0.01	5.29±0.02
Cheese packed day 11 at °2C	5.23±0.09	5.27±0.01
Cheese packed day 11 at °8C	5.12±0.01	5.12±0.01
Cheese packed day 11 at 2/8°C	5.21±0.01	5.21±0.01
Cheese packed day 15 at °2C	5.24±0.08[a]	5.44±0.10[b]
Cheese packed day 15 at °8C	5.17±0.01[a]	5.35±0.06[b]
Cheese packed day 15 at 2/8°C	5.16±0.01[a]	5.43±0.03[b]
Water activity		
Raw materials and semi-finished products		
Milk before pasteurisation	0.9966±0.0009	1.0016±0.0018
Milk post pasteurisation	0.9972±0.0005	1.0026±0.0009
Calf rennet	0.8262±0.0007	0.8663±0.0043
Cheese at the end of the storage in warm room	0.9911±0.0021	0.9957±0.0012
Cheese at the end of maturation	0.9921±0.0007	0.9962±0.0030
Final products		
Cheese after packaging	0.9916±0.0004	0.9949±0.0020
Cheese packed day 1 at °2C	0.9914±0.0004	0.9942±0.0011
Cheese packed day 1 at °8C	0.9909±0.0004	0.9944±0.0016
Cheese packed day 1 at 2/8°C	0.9913±0.0003	0.9941±0.0011
Cheese packed day 4 at °2C	0.9908±0.0004	0.9945±0.0030
Cheese packed day 4 at °8C	0.9912±0.0002	0.9950±0.0015
Cheese packed day 4 at 2/8°C	0.9919±0.0007	0.9982±0.0038
Cheese packed day 8 at °2C	0.9917±0.0020	0.9963±0.0013
Cheese packed day 8 at °8C	0.9896±0.0025	0.9969±0.0018
Cheese packed day 8 at 2/8°C	0.9922±0.0003	1.0016±0.0040
Cheese packed day 11 at °2C	0.9950±0.0005	1.0014±0.0027
Cheese packed day 11 at °8C	0.9952±0.0002	1.0017±0.0011
Cheese packed day 11 at 2/8°C	0.9948±0.0006	1.0005±0.0068
Cheese packed day 15 at °2C	0.9935±0.0009	0.9949±0.0005
Cheese packed day 15 at °8C	0.9956±0.0011	0.9959±0.0004
Cheese packed day 15 at 2/8°C	0.9970±0.0008	0.9965±0.0006

Table 2: Microbial growth (lactic acid bacteria and total bacteria count express in $\log_{10}$ CFU/g or cm^2) and physicochemical values (pH and water activity) in samples of raw materials, semi-finished and finished products and the environment of soft cheese, across the two tested batches. Different superscript letters indicate significant differences in microbial counts between batches ($p \leq 0.05$).

Lactic acid bacteria (LAB)		
Sample	**Summer batch**	**Winter batch**
Raw materials and semi-finished products		
Raw meat	3.37 ± 0.06a	3.13 ± 0.27a
Salami stored 1 week in drying room	8.53 ± 0.07def	6.59 ± 0.08b

Salami at 3 weeks of ripening	9.08 ± 0.31ef	8.37 ± 0.33de
Salami at 10 weeks of ripening	9.34 ± 0.45f	8.28 ± 0.18de
Salami at 18 weeks of ripening	8.48 ± 0.35de	7.04 ± 0.07bc
Final products		
Salami at 28 weeks of ripening	7.78 ± 0.49cd	7.83 ± 0.11cd
Total bacteria count (TBC)		
Raw materials and semi-finished products		
Raw meat	4.04 ± 0.03a	4.69 ± 0.25a
Salami stored 1 week in drying room	8.58 ± 0.11cd	6.78 ± 0.12b
Salami at 3 weeks of ripening	8.96 ± 0.18cde	8.31 ± 0.12cd
Salami at 10 weeks of ripening	9.75 ± 0.80e	8.35 ± 0.16cd
Salami at 18 weeks of ripening	9.13 ± 0.10de	8.72 ± 0.13cde
Final products		
Salami at 28 weeks of ripening	8.77 ± 0.27cde	8.25 ± 0.07c
Environmental samples		
Manhole in mixture room	7.68 ± 0.08f	5.79 ± 0.49de
Table in mixture room	4.28 ± 0.46bc	4.07 ± 0.05abc
Filler stuffer machine in mixture room	3.25 ± 0.86ab	2.66 ± 0.46a
Walls in drying room	4.18 ± 0.35bc	4.25 ± 0.16bc
Manhole in drying room	6.80 ± 0.05ef	6.85 ± 0.20ef
Walls in ripening room	6.47 ± 0.18def	5.19 ± 0.71cd
Manhole in ripening room	6.82 ± 0.36ef	7.04 ± 0.64ef
Enterobacteriaceae		
Raw materials and semi-finished products		
Raw meat	4.63 ± 0.35fg	3.19 ± 0.13fg
Salami stored 1 week in drying room	4.56 ± 0.14fg	5.49 ± 0.29fg
Salami at 3 weeks of ripening	4.11 ± 0.10ef	4.84 ± 0.55ef
Salami at 10 weeks of ripening	4.41 ± 0.10f	2.18 ± 0.77f
Salami at 18 weeks of ripening	<1.00 + 0.00a	1.60 ± 0.50a
Final product		
Salami at 28 weeks of ripening	1.09 ± 0.24a	<1.00 ± 0.00a
pH		
Raw materials and semi-finished products		
Raw meat	5.67 ± 0.02c	5.73 ± 0.06c
Salami stored 1 week in drying room	5.28 ± 0.15a	5.58 ± 0.00bc
Salami at 3 weeks of ripening	5.41 ± 0.01ab	5.46 ± 0.00ab
Salami at 10 weeks of ripening	5.41 ± 0.03ab	5.77 ± 0.01c
Salami at 18 weeks of ripening	5.92 ± 0.03d	5.95 ± 0.04d
Final product		
Salami at 28 weeks of ripening	6.26 ± 0.01e	6.02 ± 0.03d
Water activity		
Raw materials and semi-finished products		
Raw meat	0.9816 ± 0.0047f	0.9810 ± 0.0017f
Salami stored 1 week in drying room	0.9632 ± 0.0035e	0.9717 ± 0.0000ef
Salami at 3 weeks of ripening	0.9597 ± 0.0775e	0.9597 ± 0.0000e
Salami at 10 weeks of ripening	0.9498 ± 0.0006d	0.9125 ± 0.0036c
Salami at 18 weeks of ripening	0.8647 ± 0.00939a	0.9102 ± 0.0020c

Final product		
Salami at 28 weeks of ripening	0.8843 ± 0.0030b	0.8844 ± 0.0029b

Table 3: Microbial growth (lactic acid bacteria, total bacteria count and *Enterobactericeae* express in log_{10} CFU/g or ml or cm^2) and physicochemical values (pH and water activity) in samples of raw materials, semi-finished and finished products and the environment of salami, across the two tested batches. Different superscript letters indicate significant differences in microbial counts between batches ($p \leq 0.05$).

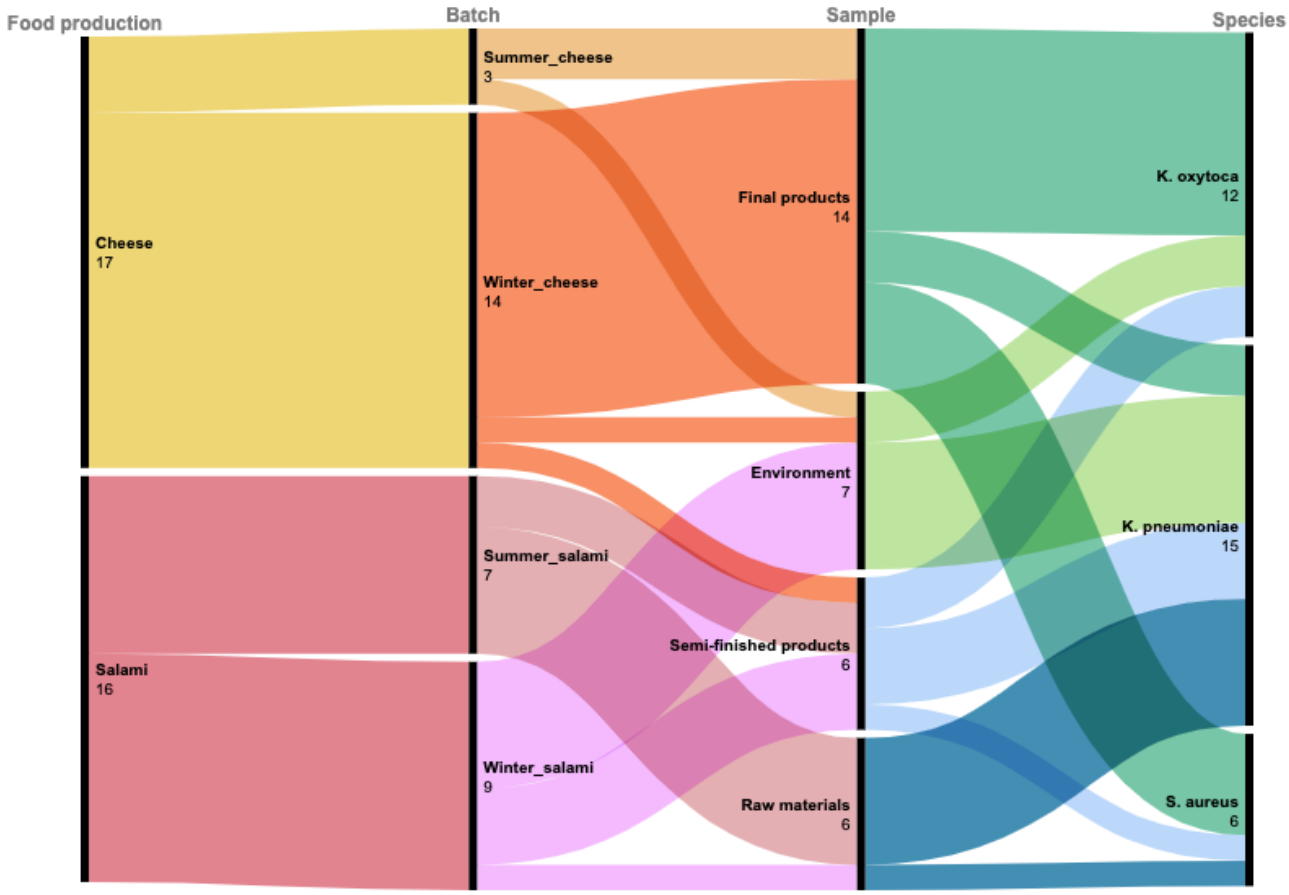


Figure 1: Alluvional diagram showing biological hazards confirmed with biochemical and molecular (PCR) test in the selected traditional productions of cheese and salami, plotted with RAWGraphs 2.0 (https://app.rawgraphs.io/)

Sample	*Klebsiella oxytoca*	*Klebsiella pneumoniae*	*Staphylococcus aureus*
Cheese			
Summer batch			
Final products			
Cheese packed day 0	Detected (n=1)		
Cheese packed day 4 - 2°C		Detected (n=1)	
Environment			
Maturation room wall swab	Detected (n=1)		
Winter batch			
Semi-finished products			
Cheese at the end of maturation	Detected (n=1)		
Final products			
Cheese packed day 0	Detected (n=1)		
Cheese packed day 1 - 2°C	Detected (n=1)		Detected (n=1)
Cheese packed day 1 - 8°C	Detected (n=1)		
Cheese packed day 4 - 2°C	Detected (n=1)		Detected (n=1)
Cheese packed day 4 - 2/8°C	Detected (n=1)		
Cheese packed day 8 - 2°C			Detected (n=1)
Cheese packed day 11 - 2°C	Detected (n=1)		Detected (n=1)
Cheese packed day 11 - 8°C	Detected (n=1)		
Cheese packed day 15 - 2°C		Detected (n=1)	
Environment			
Manhole within the maturation room	Detected (n=1)		
Total positive samples	**n=11**	**n=2**	**n=4**
Salami			
Summer batch			
Raw materials			
Raw meat mixture		Detected (n=4)	Detected (n=1)
Semi-finished products			
Salami stored for 1 week inside drying area	Detected (n=1)	Detected (n=1)	
Winter batch			
Raw materials			
Raw meat mixture		Detected (n=1)	
Semi-finished products			
Salami stored for 1 week inside drying area		Detected (n=1)	Detected (n=1)
Salami stored for 3 weeks inside ripening area		Detected (n=1)	
Environment			
Processing surfaces inside stuffing area		Detected (n=1)	
Filler stuffer machine inside stuffing area		Detected (n=1)	
Manhole inside stuffing area		Detected (n=3)	
Total positive samples	**n=1**	**n=13**	**n=2**

Table 4: Full metadata description (food product, batch, origin and matrices of samples) of *K. oxytoca*, *K. pneumoniae* and *S. aureus* hazards in the selected traditional productions.

Discussion

In the present study, the microbial quality and safety were investigated in Italian artisanal cheese and salami processed in summer and winter seasons, by monitoring the microbial load of hygiene indicator bacterial groups together with biological hazards through the processing environments and whole production chains. Starting from cheese, the fall of TBC along with the absence of *Enterobacteriaceae* and pathogens in pasteurized milk suggested that for both batches the heat treatment was capable to kill all pathogenic bacteria while reducing the number of organisms that can possibly cause spoilage. *Enterobacteriaceae* values showed accordance with European Regulation on microbiological criteria (EC 2073/2005) for pasteurized milk (<1 MPN/ml). As expected, during

further cheesemaking processes, such as the inclusion of the starter *Streptococcus thermophilus*, both TBC and LAB increased (Vrdoljak et al., 2016). Interestingly, for this artisanal production seasonality's variation is liable to influence the bacterial growth, suggesting a boost of TBC and LAB in winter corresponding to the end of the cheese storage exposed to abuse temperatures. Moreover, the winter seasonality had a significant effect also for physicochemical attributes, being associated to higher values of pH in rennet and in cheese during maturation and storage when compared to summer. This seasonal variability has been already suggested for an Italian artisanal cheese from goat milk, where mesophilic bacteria, LAB and pH values were higher for cheese samples produced in wintertime (Pino et al., 2021). A possible explanation to the rise in LAB counts might be seasonal variations in milk fat composition which may have endorsed LAB growth performances (Larsen et al., 2014; Tan et al., 2012). Similarly, being the cheese pH usually affected by several factors encompassing the formation of organic acids (e.g., lactic acid) as well as the content of calcium, phosphorous and lactose, it is possible that their shift influenced pH changes (Upreti & Metzger, 2007). However, it has been previously observed that the rennet concentration was responsible of increased pH of cheese during ripening (Soodam et al., 2015). Nonetheless, a further study on the sugar and mineral content of the cheese may bring more insight on the highlighted seasonal variation. Overall, LAB and TBC values of soft cheese investigated in this study (LAB from 1.14 $\log_{10}$ CFU/g at day 0 to 5.31 $\log_{10}$ CFU/g at day 15; TBC from 5.46 $\log_{10}$ CFU/g at day 0 to 8.03 $\log_{10}$ CFU/g at day 15) showed microbial counts comparable or lower to other similar soft cheeses (LAB from 5 to 8 $\log_{10}$ CFU/g; TBC from 6 to 8 $\log_{10}$ CFU/g) (Vrdoljak et al., 2016; Gérard et al., 2020; Ortolani et al., 2010).

Across the salami artisanal production, the raw materials represented by meat mixture exhibited TBC values (4.04 and 4.69 $\log_{10}$ CFU/g) below the microbiological criteria (EC 2073/2005) for minced meat ($<5 \times 10^5$ CFU/g).

For both summer and winter, the long ripening period of about 6 months has been associated to the LAB and TBC increase, alongside a reduction of *Enterobacteriaceae* and water activity, independently from the analysed batch. This trend was previously reported by Settanni et al. (2020) for Italian spontaneously fermented pork salami. Moreover, comparable values were reported between salami and similar dry fermented sausages products concerning LAB (7.8 vs. 7.9 $\log_{10}$ CFU/g), as well as TBC (8.5 vs. 8.2 $\log_{10}$ CFU/g) (Barbieri et al., 2018; Rubio et al., 2007). Physicochemical properties of salami did not compromise microbial quality; water activity reached at the end of ripening (≤0.88) are under the minimum required for growth of most bacteria (Coroller et al., 2015; Novelli et al., 2017). Moreover, the final slight increase of pH during ripening was observed in other reports (Casaburi et al., 2007; Cenci-Goga et al., 2008; Settanni et al., 2020). The investigated artisanal salami has been produced without the addition of nitrate, nitrite and starter cultures, thus the fermentation process depended exclusively on the natural evolution of indigenous starter cultures. Regarding seasonality influence, during the summer ripening phase LAB and TBC have been likely to multiplicate at higher levels, probably as a consequence of environmental conditions.

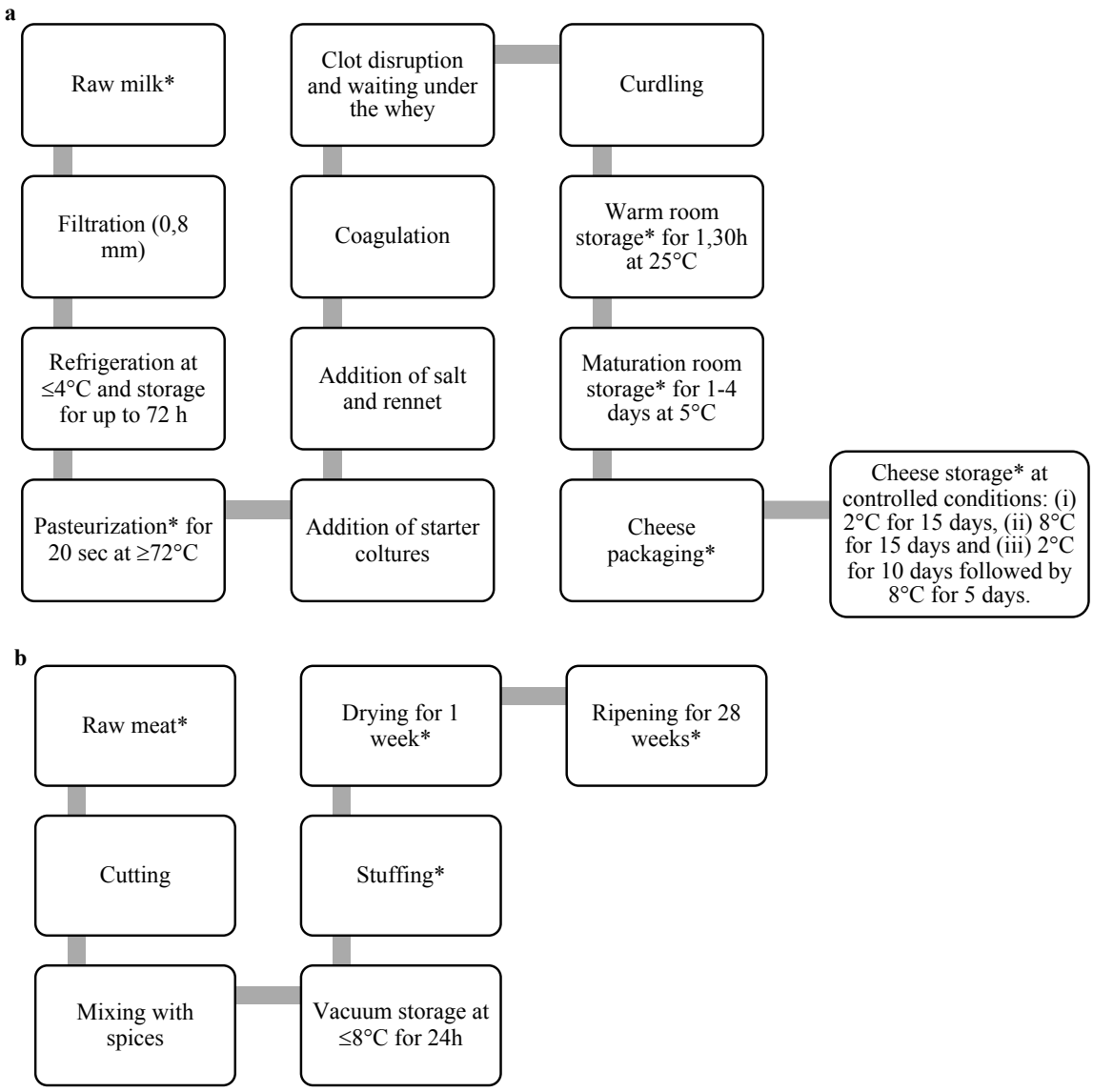
Regarding microbial hazards, neither cheese nor salami represented a vehicle of foodborne pathogens such as *L. monocytogenes*, *Salmonella* or VTEC, thereby suggesting that the combination of hurdles during artisanal manufacturing have blocked the growth of these concerning hazards. In cheese, the combination of thermal milk treatment and the use of bio-protective cultures avoided pathogens proliferation and persistence, whereas is salami hurdles were linked to the autochthonous microbial populations and technological parameters. Nevertheless, *S. aureus* and *Klebsiella* spp. were detected. The presence of *S. aureus* in the final product obtained from the winter batch cheese may be connected to a general worsen microbiological quality (e.g., higher pH) and addresses concerns for consumer's safety, although further studies are needed to explore their pathogenic traits. Likewise, *K. oxytoca* and *K. pneumoniae,* mainly associated to cheese and salami respectively, were spread along both artisanal chains over multiple seasonality, from raw materials to semi-finished and final products as well as the environment.

The identification of *K. pneumoniae* and *K. oxytoca* is of relevance since these bacteria are pathogens of clinical importance, generally acquired by environmental sources and are often associated to bronchopneumonia, urinary tract infection and septicaemia in hospitalized patients (Gómez et al., 2021). Although *Klebsiella* spp. has been previously isolated from cheese and meat products (Barbieri et al., 2021; Kongo et al., 2008; Massa et al., 1992; Roig-Sagues et al., 1996; Theocharidi et al., 2022; Tornadijo et al., 1993; Uraz et al., 2008), to the best of author's knowledge yet no *Klebsiella* human infection has been associated to consumption of contaminated food, although this possibility cannot be ruled out. Further investigations should be performed on *Klebsiella* spp. retrieved from this study, to evaluate the origin of food contamination (i.e., raw materials, personnel, processing environment) as well as their antimicrobial susceptibility, and potential pathogenicity.

Conclusions

The investigated artisanal food cheese and meat productions did not present any risk connected to the presence of *L. monocytogenes*, *Salmonella* or VTEC. In cheese, the winter season production has been linked to higher microbial growth of LAB and TBC together with higher pH values, which might have promoted the dissemination of *S. aureus* and *K. oxytoca.* The artisanal salami manufacturing lacking the use of cultures starters and nitrates exhibited overall positive performance of the autochthonous microbial consortia and other hurdles in preserving the microbial quality of final ready-to-eat products. Nonetheless, the isolation of *S. aureus* and *K. pneumoniae* during salami processing and environmental sites as well as *S. aureus* and *K. oxytoca* from cheese and its environment suggests the need to focus attention on improving the processing standardization and strengthening hygienic procedures. Further studies should be carried out to investigate the potential pathogenicity as well as environmental transmission of isolated strains.

Supplementary material



Supplementary Figure 1: Flowcharts summarizing key processing stages in cheese (a) and salami (b) productions realized in the artisanal plants. Processing areas and stages where samples from food (raw materials, semi-finished and final products) and processing environment were taken have been marked by adding an asterisk within the boxes.

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   Title: Investigation on the microbiological hazards in an artisanal soft cheese produced in northern Italy and its production environment in different seasonal periods

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   Title: Investigation on the microbiological hazards in an artisanal salami produced in Northern Italy and its production environment in different seasonal periods

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Study 2

Genomic snapshot of foodborne pathogens from artisanal food productions of animal origin in the Mediterranean region: occurrence, resistome and virulome

Abstract

The aim of the present study was to assess the presence and investigate AMR and virulence key traits of *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* strains from artisanal fermented meat and dairy productions typically consumed in four Countries belonging to the Mediterranean area (Portugal, Spain, Italy and Morocco). By implementing a WGS approach, *in silico* 7-loci MLST and SNP calling were carried out to assess strains phylogenetic relationships whereas Abricate was used to explore resistome and virulome. Over 2,800 samples were retrieved from raw materials, semi-finished, final products and environmental sites of the artisanal facilities in 4 to 6 tested batches (Italian dairy and meat samples were collected from the two batches described in study 1 and four additional batches). Fourty-two isolates were confirmed as *L. monocytogenes* (n=14)*, S. enterica* (n=13), and *S. aureus* (n=15). Among each species, STs diversity and distribution indicated the circulation of different clones in the same artisanal production plant over several months, thereby confirming the ability of these pathogens to contaminate, spread and persist in the artisanal productions. Many clones of *L. monocytogenes* disseminated among Spanish and Italian artisanal meat facilities, showing concerning Sequence Types (ST1 and ST8) and carrying several pathogenic features, such as the full-length *inlA* gene*,* the Listeria Pathogenicity Island 1 (LIPI1) and 3 (LIPI3), through the environment and fermented Spanish sausage. Likewise, *S. enterica* was identified in meat facilities from Portugal and Morocco. Although five different serovars contaminated Moroccan fermented sausage, one clone of *S*. Paratyphi B ST43 persisted in the Portuguese meat processing environment and final products over one year, exhibiting the highest repertoire of virulence genes (pathogenicity islands SP1, SP2, SP3 and other virulence markers). *S. aureus* showed the highest diversity in terms of country of isolations (Spain, Italy and Morocco), food productions (cheese and meat) and STs distribution (six known and two novel STs found across raw materials, semi-finished and final products). This diversity suggested the occurrence of several cross-contamination events driving *S. aureus* dissemination in artisanal facilities over time, with ST121 carrying the higher number of virulent traits. None of genomes but three *S. enterica* serovar Hadar and Seftemberg carried genes associated to resistance to aminoglycosides, beta-lactams, sulphonamides, trimethoprim and tetracyclines. Overall, the occurrence of virulent and hypervirulent *L. monocytogenes, S. enterica* and *S. aureus* strains in artisanal fermented meat and dairy productions suggest to strengthening prevention and control measures aimed at reducing these biological hazards in the artisanal food chain and safeguard consumers health.

Introduction

Thus far, studies related to AMR and virulence traits in food of animal origin have largely focused on commercial-scale production systems while lesser insights have been addressed to artisanal small-scale productions (Graham et al., 2017).

Recent years have discerned an increased consumer demand for artisanal foods and beverages. These products are generally obtained from small-scale local productions and are perceived as healthier and more genuine compared to their industrial counterparts, resulting in growing consumer's attractiveness and popularity (Capozzi et al., 2020; Frizzo et al., 2020). On the other hand, small-scale productions are often less standardized, lack full developed Hazard Analysis and Critical Control Points approach, and may embrace a higher product handling by staff personnel than industrial ones. In these conditions, several challenges and limitations are addressed in management and control of production parameters and biological hazards prevention (Ditlevsen et al., 2020; Halagarda & Wójciak, 2022; Tamang et al., 2020).

Pathogenic bacteria exhibiting AMR patterns are increasingly found in foods of animal origin, leading to their possible dissemination through the food chain (Alsayeqh et al., 2021). The spread of foodborne antibiotic-resistant microorganisms has been recently acknowledged by the World Health Organization as one of the top ten threats to public health and food safety (WHO, 2021). More worryingly, the coexistence of AMR and virulence factors, which allow a microorganism to establish itself on or within a host and thus increase its ability to cause disease, has inevitably fostered clinical outbreaks, thereby resulting in potentially fatal infections and serious public health threats (Gu et al., 2018; Kalil et al., 2014).

Within the framework of H2020 PRIMA European funded project ARTISANEFOOD (http://www.ipb.pt/artisanefood/), partners from Portugal, Spain, Italy, and Morocco identified local, artisanal fermented food products of dairy and meat origins. In order to gain a snapshot of the sanitary and hygienic status of these productions, sample were collected from raw materials, intermediate and finished products as well as the environment. To expand current knowledge on AMR and virulence dissemination through the artisanal production chain, *L. monocytogenes*, *S. enterica* and *S. aureus* isolates, acknowledged as major foodborne pathogens of public health significance, were investigated in this study for their resistome and virulome, in order to elucidate their AMR and pathogenic diversity and potential dissemination within small-scale production facilities.

Materials and methods

Sampling overview

The sampling took place in four different Mediterranean countries: Spain, Italy, Portugal and Morocco. In each country local meat and dairy artisanal productions were selected as follows: Spain (cheese from sheep's milk and raw-cured pork sausage "salchichón"), Italy (soft cheese from pasteurized cow's milk and salami of pork origin), Portugal (cheese from raw goat's milk and Alheira pork/poultry sausage) and Morocco (merguez beef/lamb sausage). For each production, samples of

raw materials, semi-finished and finished fermented products, as well as from food contact surfaces were collected along with the production environment according to the methods reported by Pasquali et al. (2022). In each artisanal production, from 12 to 15 samples, with two to five replicates each, were overall collected in four to six batches over a time-line continuum production ranging from 2,800 samples. Details on processing workflows and samples collection from Italian productions are provided in Study 1 (Supplementary Figure 1) with the exception of the number of tested batches which were two in study 1 (winter and summer) and 6 in study 2.
Overall, a total of approx. 2,800 samples were collected and submitted to ISO standard methods as well as biochemical and PCR tests for the isolation and identification of *L. monocytogenes*, *Salmonella enterica*, *S. aureus* and VTEC, as previously described (Pasquali et al., 2022).

Whole Genome Sequencing and bioinformatic analyses

After biochemical and/or molecular confirmation, *L. monocytogenes*, *Salmonella enterica* and *S. aureus* isolates were submitted to DNA extraction using MagAttract HMW DNA Kit (Quiagen, Hilden, Germany). Libraries were built using the Nextera DNA sample Prep Kit (Illumina, Milan, Italy), following paired-end sequencing by Illumina MiSeq platform (Milan, Italy).
Raw reads of 250 bp on average were firstly submitted to RefSeq Masher Matches v0.1.2 for species confirmation (https://github.com/phac-nml/refseq_masher), by filtering bacterial species other than *L. monocytogenes*, *Salmonella enterica* and *S. aureus* for further downstream analyses. *De novo* assembly was then carried out with Unicycler v0.5.0, which includes Spades v3.14.0 assembler (https://github.com/rrwick/Unicycler), and assemblies quality checked by QUAST v5.2.0 (https://github.com/ablab/quast).
The phylogenetic analysis of genomes was performed by *in silico* MLST v2.22.0 (https://github.com/tseemann/mlst) and Snippy v4.6.0 (https://github.com/tseemann/snippy) on assembled contigs and raw reads respectively. Concerning SNPs analysis, the most represented ST-type were selected as reference for each species, to call nucleotides variants between closely related genomes (Besser et al., 2018). Genomes ArFCLM01 and ArFASE04 were taken as references for *L. monocytogenes* and *Salmonella enterica* respectively, whereas for *S. aureus* the public reference genome ASM1342v1 (RefSeq GCF_000013425.1) was selected due to the high diversity of the population in term of ST-types, country and food origins. After variants calling, a maximum likelihood (ML) phylogeny was inferred on core SNP alignments using PhyML v3.1 (https://github.com/stephaneguindon/phyml), and phylogenetic trees were visualized and annotated with iTOL v6 (Letunic & Bork, 2021). A pairwise SNP distance matrix was generated using snp-dists v0.6.3 (https://github.com/tseemann/snp-dists).
Analyses of the resistome and virulome of all genomes were performed using ABRicate v1.0.1, by selecting Resfinder (Zankari et al., 2012) and VFDB (Chen et al., 2016) public databases (https://github.com/tseemann/abricate). Heatmaps were then generated with ggplot2 package in R v4.1.2 (https://cran.r-project.org/).
Sequencing data are available at NCBI Database under BioProject Accession number PRJNA876122.

Results and discussion

Over 2,800 samples collected, a total of 42 isolates were confirmed as *L. monocytogenes* (n=14), *S. enterica* (n=13), and *S. aureus* (n=15) (Table 1). Overall, pathogens identifications showed a higher prevalence of these hazards from meat-derived artisanal productions (86%; 37 out of 43) compared to cheese, which tested as positive only for *S. aureus*.

Sample code	Sample	Country	Isolation matrix*	Isolation date	Species	ST-type	Antimicrobial resistance associated genes
ArFCLM01	LM1	Spain	sausage – FP	20/07/2020	*L.monocytogenes*	ST3	*fosX*
ArFCLM02	LM2	Spain	sausage – FP	20/07/2020	*L.monocytogenes*	ST3	*fosX*
ArFCLM03	LM3	Spain	sausage -RM	15/09/2020	*L.monocytogenes*	ST1	*fosX*
ArFCLM04	LM5	Spain	sausage-E	15/09/2020	*L.monocytogenes*	ST8	*fosX*
ArFCLM05	LM6	Spain	sausage-E	30/09/2020	*L.monocytogenes*	ST451	*fosX*
ArFCLM06	LM7	Spain	sausage - RM	20/10/2020	*L.monocytogenes*	ST1	*fosX*
ArFCLM07	LM8	Spain	sausage – FP	20/10/2020	*L.monocytogenes*	ST8	*fosX*
ArFCLM08	LM9	Spain	sausage – FP	20/10/2020	*L.monocytogenes*	ST1	*fosX*
ArFCLM09	LM13	Spain	sausage – E	20/10/2020	*L.monocytogenes*	ST8	*fosX*
ArFCLM10	LM14	Spain	sausage – E	20/10/2020	*L.monocytogenes*	ST8	*fosX*
ArFFLM01	2SWD2A	Italy	salami – E	29/09/2020	*L.monocytogenes*	ST489	*fosX*
ArFFLM02	2SWD2B	Italy	salami – E	29/09/2020	*L.monocytogenes*	ST489	*fosX*
ArFFLM03	2SWD5A	Italy	salami - E	29/09/2020	*L.monocytogenes*	ST489	*fosX*
ArFFLM04	2SWD5B	Italy	salami - E	29/09/2020	*L.monocytogenes*	ST489	*fosX*
ArFASE02	S1-1A	Portugal	sausage – FP	28/01/2021	*S*. Paratyphi B	ST43	*aac(6')-Iaa*
ArFASE03	S1-1B	Portugal	sausage- E	10/11/2019	*S*. Paratyphi B	ST43	*aac(6')-Iaa*
ArFASE04	S1-2A	Portugal	sausage – FP	28/01/2021	*S*. Paratyphi B	ST43	*aac(6')-Iaa*
ArFASE05	S2-1A	Portugal	sausage – E	10/11/2019	*S*. Paratyphi B	ST43	*aac(6')-Iaa*
ArFASE06	S2-2C	Portugal	sausage – E	10/11/2019	*S*. Paratyphi B	ST43	*aac(6')-Iaa*
ArFASE07	S2-3B	Portugal	sausage – E	10/11/2019	*S*. Paratyphi B	ST43	*aac(6')-Iaa*
ArFASE11	S4-3C	Portugal	sausage – E	08/12/2019	*S*. Paratyphi B	ST43	*aac(6')-Iaa*
ArFMSE01	SALM1	Morocco	sausage – FP	22/10/2019	*S*. Hadar	ST33	*aac(6')-Iaa, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA12, sul1, tet(A)*
ArFMSE02	SALM10	Morocco	sausage – FP	14/01/2020	*S*. Kentucky	ST314	*aac(6')-Iaa*
ArFMSE03	SALM2	Morocco	sausage – FP	22/10/2019	*S*. Montevideo	ST3667	*aac(6')-Iaa*
ArFMSE04	SALM24	Morocco	sausage – FP	13/10/2020	*S*. Hadar	ST33	*aac(6')-Iaa, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA12, sul1, tet(A)*
ArFMSE05	SALM25	Morocco	sausage – FP	13/10/2020	*S*. Albany	ST1818	*aac(6')-Iaa*
ArFMSE06	SALM3	Morocco	sausage – FP	22/10/2019	*S*. Seftemberg	ST198	*aac(6')-Iaa, aadA2, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA12, sul1, tet(A)*
ArFCSA01	SA18	Spain	sausage – RM	29/06/2020	*S. aureus*	ST15	*blaZ, tet(K)*
ArFCSA02	SA19	Spain	sausage - RM	29/06/2020	*S. aureus*	ST15	*blaZ, tet(K)*
ArFCSA03	SA20	Spain	sausage - RM	29/06/2020	*S. aureus*	-	*blaZ, tet(K)*

ArFCSA04	SA25	Spain	sausage-FP	15/09/2020	*S. aureus*	ST7	-
ArFCSA05	SA33	Spain	sausage-FP	20/10/2020	*S. aureus*	ST7	-
ArFCSA06	SA34	Spain	sausage-FP	20/10/2020	*S. aureus*	ST7	-
ArFCSA07	SA48	Spain	sausage - RM	31/08/2020	*S. aureus*	ST8	-
ArFCSA08	SA49	Spain	sausage - RM	31/08/2020	*S. aureus*	ST8	-
ArFDSA01	SA101	Spain	cheese - FP	22/09/2020	*S. aureus*	ST121	*blaZ, str*
ArFDSA03	SA103	Spain	cheese - FP	22/09/2020	*S. aureus*	ST121	*blaZ, str*
ArFDSA04	SA105	Spain	cheese – RM	18/02/2020	*S. aureus*	ST398	*blaZ, tet(M)*
ArFESA01	L2 CP1582	Italy	cheese – FP	05/06/2020	*S. aureus*	-	-
ArFESA03	L6 CP11285	Italy	cheese – FP	15/03/2021	*S. aureus*	ST8	*blaZ*
ArFFSA03	6SBR4	Italy	salami - SFP	09/12/2020	*S. aureus*	ST5	*blaZ*
ArFMSA01	ST.AU.3	Morocco	sausage - FP	22/10/2019	*S. aureus*	ST15	*blaZ, tet(K)*

*FP: finished product, SFP: semifinished product, RM: raw material (minced meat or milk), E: production environment

Table 1: Isolation country, matrix, date and detected AMR genes of the 42 confirmed strains collected from the tested artisanal facilities.

Listeria monocytogenes

Among the 14 *L. monocytogenes* identified, ten samples were associated to the Spanish salchichón production, isolated from raw meat, environment and final product over four months. The four remaining strains were collected only from the processing environment of Italian salami, without proving a persistence on the plant and transmission to final products (Table 1). These strains were collected from the same batch belonging to the fourth batches analysed in addition to the two of study 1.

Statistics of *de novo* assemblies showed good quality of sequenced genomes, with number of contigs ranging from 25 to 32, genome size from 2.7 to 3.0 Mb, GC content of 37% and N50 from 408574 to 533374 (Supplementary Table 1).

By MLST analysis, five STs were distinguished, such as ST3, ST1, ST8, ST451 and ST489. ST8, ST1 and ST3 were the most represented among the Spanish production, whereas ST489 belonged to Italian isolates. Core SNPs phylogenetic tree (Figure 1) showed that genomes clustered according to the country of origin and STs, highlighting that ST1 and ST8 clades encompassed strains isolated from different origins of samples (environment, raw materials and final products) and reported between 65-73 and 599-739 SNPs differences respectively (Supplementary Table 2). Both ST1 and ST8 have been frequently associated to clinical listeriosis, thus raising concerns on the potential transmission to humans of newly sequenced isolates through food consumption (Amato et al., 2017; Ge et al., 2022; Lu et al., 2021).

Regarding AMR prediction, all genomes carried the intrinsic fosfomycin resistance (*fosX*) gene, no further AMR genes were detected, suggesting the low AMR burden (Table 1).

Moving to virulence features (Figure 2), all fourteen *L. monocytogenes* genomes but one (ArFCLM05, ST451) showed a full-length *inlA* gene, which is an essential virulence factor that allows to cross the intestinal barrier and facilitate the invasion of human intestinal epithelial cells (Su et al., 2019).

Listeria Pathogenic Islands 1 (LIPI 1) was detected in all genomes, however, ST1, one of the most represented in Spanish production, lacked *actA* gene generally located in LIPI 1 along with *prfA, actA, hly, mpl, plcA, plcB*, and *iap* genes. In addition, ST1, ST3 and ST489 genomes carried LIPI 3 (*llsA, llsG, llsH, llsX, llsB, llsY, llsD, llsP*), which has been associated, together with LIPI 1, to increased virulence and invasiveness (*llsX* gene of LIPI 3) (Vilchis-Rangel et al., 2019).

Taken together, phylogenetic tree clustering along with SNP distance values, STs and resistance/virulence prediction, suggested that different isolates of *L. monocytogenes* were circulating in the Spanish raw-cured sausage plant from July to October 2020 and spread from the environment and raw materials through the final products, were most of strains exhibited pathogenic potential.

Figure 1: ML phylogenetic tree inferred on 14 *L. monocytogenes* strains from Spanish and Italian artisanal fermented meat productions.

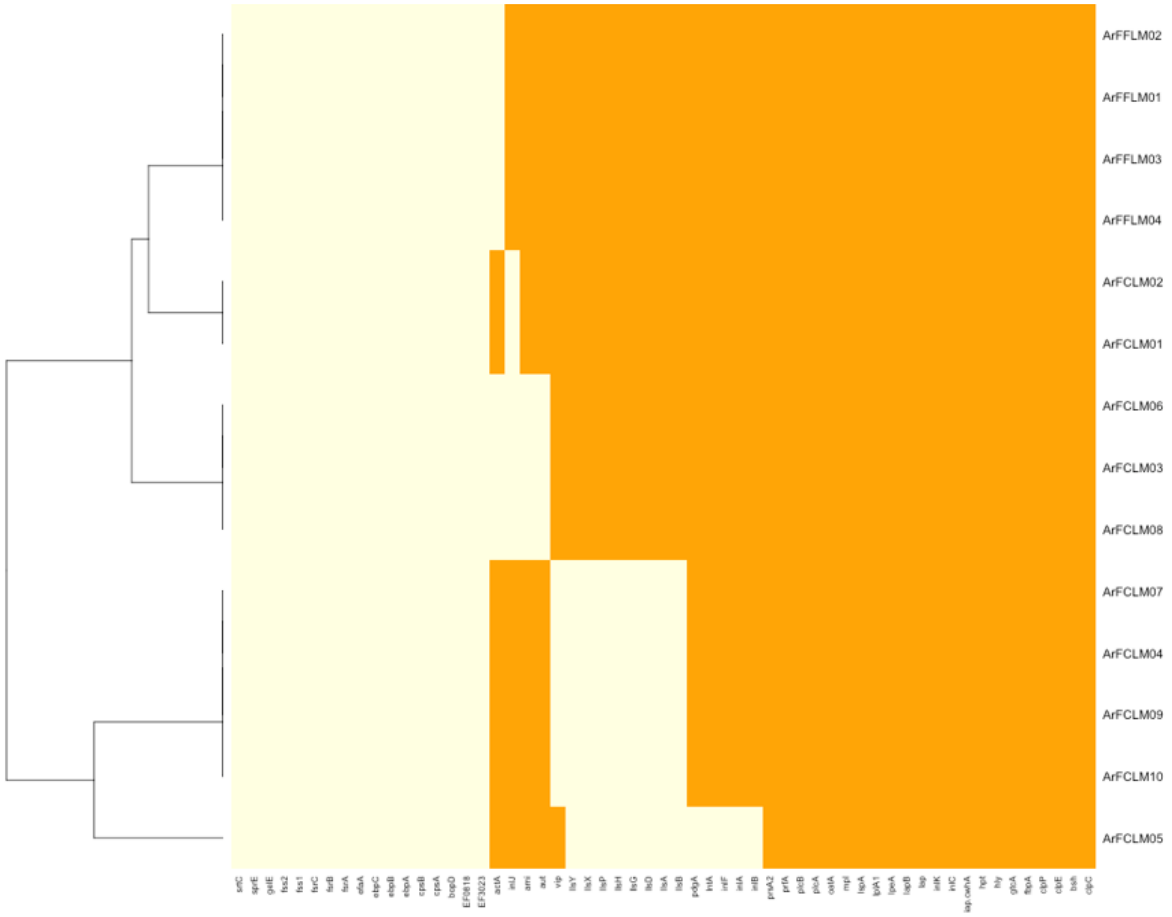

Figure 2: Heatmap of virulence features identified in 14 *L. monocytogenes* genomes. Yellow: absence (<80% of sequence identity), orange: presence (>80% of sequence identity).

Salmonella enterica

A total of 13 strains of *Salmonella enterica* were confirmed in fermented sausages from Portugal (n=7) and Morocco (n=6). Moroccan strains encompassed five serovars (Hadar, Kentucky, Montevideo, Albany and Senftemberg) found only from final products, whereas all Portuguese strains belonged to serovar Paratyphi B retrieved from the environment as well as sausages samples (Table 1). Assembled genomes showed overall good quality metrics, with number of contigs ranging from 43 to 79, genome size from 4.6 to 4.9 Mb, GC content of 52% and N50 from 231038 to 614135 (Supplementary Table 1).

Serovars variability of Moroccan strains also reflected STs diversity: ST33 was carried by *S.* Hadar, ST314 by *S.* Kentucky, ST3667 by *S.* Montevideo, ST1818 by *S.* Albany and ST198 *S.* Senftemberg. Although these serovars have been frequently isolated in poultry products, Amajoud et al. (2017) reported *S.* Kentucky, *S.* Montevideo and *S.* Hadar from meat products other than poultry in Morocco. Taking into account the origin of Moroccan sausages from this study (beef/lamb), it is feasible that some of these serovars could be related to other meat sources than poultry.

Interestingly, all *S.* Paratyphi B harbored ST43, suggesting that the same genotype persisted in the environment and contaminated the food products from November 2019 to January 2021. This hypothesis was further confirmed by SNPs phylogeny, showing that all *S.* Paratyphi B shared the

same clade within the tree and diverged by one SNP maximum, thereby resulting as clones (Figure 3, Supplementary Table 3).

AMR prediction pointed out that the chromosomal located gene *aac(6')-Iaa*, encoding for resistance to aminoglycosides, was present in all isolates. Besides aminoglycosides, other resistances were found only in Moroccan strains, since *S.* Hadar genomes carried *tet*A (tetracycline resistance) and *S.* Senftemberg showed a multiresistant profile harboring *blaTEM1-B* (beta-lactams resistance), *dfrA12* (trimethoprim resistance), *sul1* (sulphonamide resistance) and *tet*A (Table 1). Moreover, a wide repertoire of virulence traits was identified (Figure 4), with all genomes carrying from 100 to 107 genes. Although genes associated to *Salmonella* virulence plasmids were not observed, *Salmonella* pathogenicity islands SP1 (*orgABC, prgHIJK, sipABCD, sicAP, spaOPQRS, invABCEFGHIJ), SP2 (ssaGHIJKLMNOPQRSTUV, sscAB, sseABCDEFGIJK1K2L, ssaCDE*,) and SP3 (*misL, mgtBC*) were detected along with *sopE2* gene described in the literature as virulence marker of UK and Italian *S.* Typhimurium monophasic variant clades (Marcus et al., 2000; Palma et al., 2018). *S.* Paratyphi B was the serovar with the highest number of virulence genes (n=107), among which *grvA, ratB, shdA, sod C1, sseI/srfH* were not found in other serovars. Notably, *grvA* and *sodC1* have been described as part of Gifsy-2 phage contributing to the virulence of *S.* Typhimurium (Ho & Slauch, 2001).

The *S.* Paratyphi B serotype's pathogenicity is known to be characterized by different strains related with different disease outcomes. Whereas d-tartrate-nonfermenting (dT^-) strains display an enhanced human pathogenicity causing typhoid-like disease, d-tartrate-fermenting strains (dT^+), formerly designated S. *enterica* subsp. *enterica* serovar Java by Kauffmann (1955), are responsible of the less severe gastroenteric disease (Malorny et al., 2003). Considering that S. Java includes pathogens of public health importance that are frequently isolated from poultry (Donado-Godoy et al., 2015) and the origin of the Portuguese sausages (pork/poultry), further investigations have been carried out to assess the variant type harboured by the Portoguese *S.* Paratyphi B strains and thus speculate on their source and ability to cause invasive disease.

The molecular meaning for the differences in the ability to ferment d-tartrate relies in one SNP in the ATG start codon for the gene STM 3356, a putative cation transporter upstream from *ttdA* and *ttdB* responsible for d-tartrate metabolism. Sequence data revealed a nucleotide exchange from G to A within the ATG start codon of gene STM 3356 in the dT^- strains (Malorny at al., 2003). To assess if the seven *S.* Paratyphi B harboured the SNP responsible of the dT^+ or dT^- phenotypes, a nucleotide-nucleotide alignment was carried out with BLASTN v2.7.1+ (https://github.com/topics/ncbi-blast) by aligning *S.* Paratyphi B assemblies with the putative cation transporter STM3356 gene of the *S. enterica* subsp. *enterica* serovar Paratyphi B reference strain NCTC 5706 (dT^+, GenBank accession AY211490, query position 252) (Malorny et al., 2003). Alignments from all the seven genomes (Supplementary File 1, available at https://github.com/ceciliacrippa/Thesis_Supplementary_File_1_Study2 under the name "blast_alignments_SParatyphi.txt") showed a 100% nucleotide identity, thus predicting the ability of fermenting d-tartrate typical of Java serovars.

ST43 of *S.* Paratyphi B has previously been described as worldwide distributed and has been associated to human infections from Europe, The United States and South America (Castellanos et

al., 2020; Park et al., 2021; Rahman et al., 2021). Moreover, Java clone ST43, which represents a well-known cause of human infections, has been also isolated from poultry (Barua et al, 2014). This suggests poultry as an important source for *Salmonella* transmission in food, besides a possible food-to-human transmission of concerning serovars.

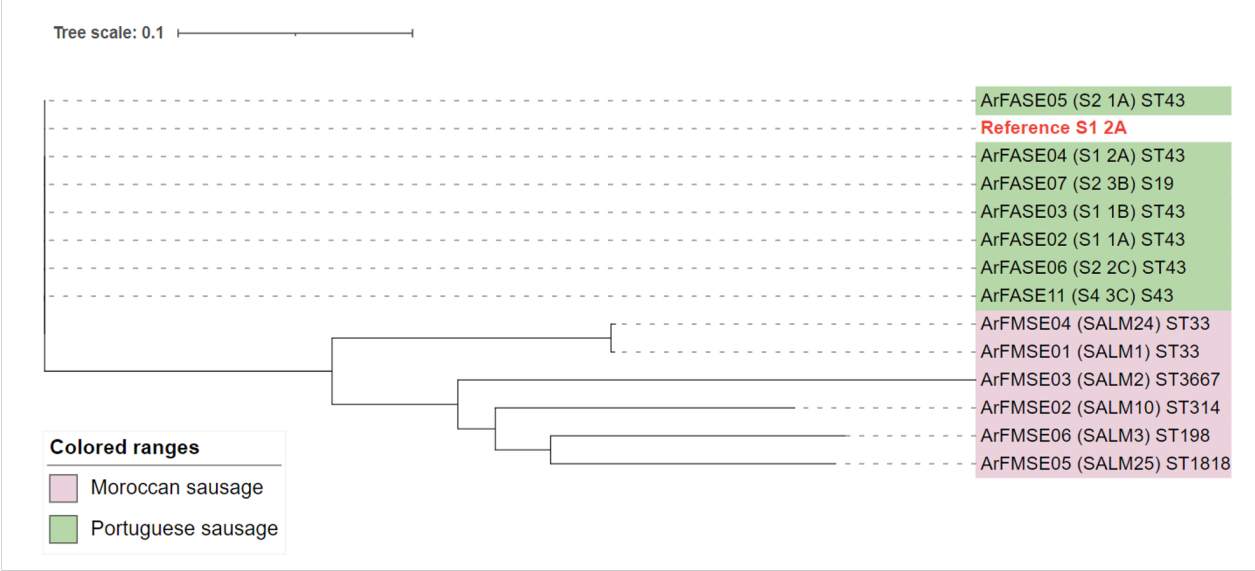

Figure 3: Core-SNPs phylogenetic tree of 13 *Salmonella* isolates from Portuguese and Moroccan artisanal productions of meat origin.

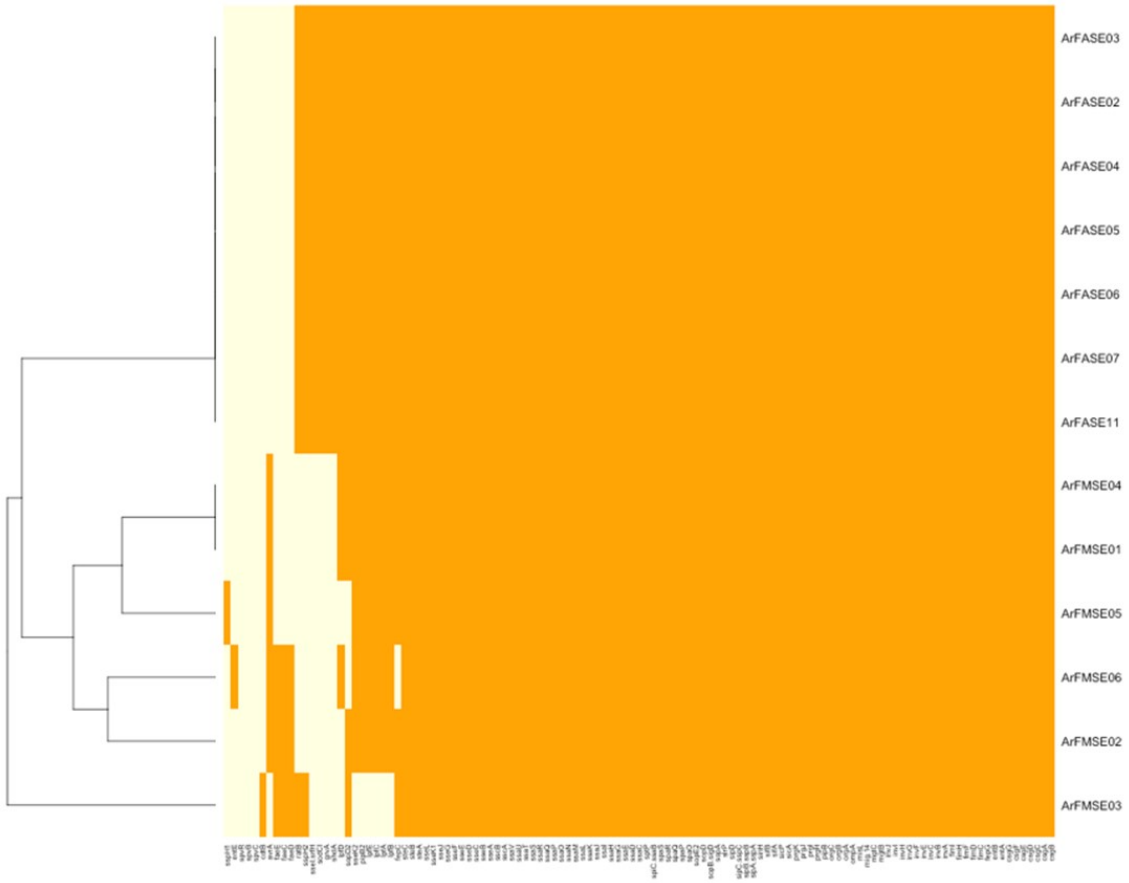

Figure 4: Virulome Heatmap belonging to 13 *Salmonella* strains. Yellow: absence (<80% of sequence identity), orange: presence (>80% of sequence identity).

Staphylococcus aureus

A total of 15 *S. aureus* isolates were confirmed from Spanish sausage and cheese (n=8 and 3 respectively), Italian cheese and salami (n=2 and 1 respectively) as well as Moroccan sausage (n=1) (Table 1). Notably, compared to *L. monocytogenes* and *S. enterica*, *S. aureus* were distributed across the artisanal food chain from a higher range of Mediterranean countries (Spain, Italy and Morocco) and food productions (cheese and meat products).

Genomes presented good assembly values, with number of contigs ranging from 24 to 49, genome size from 2.6 to 2.7 Mb, GC content of 32% and N50 from 181824 to 815868 (Supplementary Table 1).

A total of six known STs were identified and showed a strong association to food origins: ST15, ST7 and ST5 were harbored across meat samples whereas ST121 and ST398 were exclusively in cheese. The only exception was ST8, which was found in both food productions (Spanish raw sausage and Italian cheese). Furthermore, one strain from Spanish sausage (ArFCSA03) and one from Italian cheese (ArFESA01) didn't reported any known STs, suggesting the presence of novel alleles and further confirming a high genotypic diversity in terms of STs among *S. aureus* artisanal food population. As for *L. monocytogenes* and *S. enterica*, the phylogenetic tree delineated clusters mainly according to STs, food origin and country of isolation, and allowed to gain more insights into strains genetic similarity (Figure 5, Supplementary Table 4). Consistent with this, core-SNPs phylogeny revealed that ST15, ST7 and ST8 carried by Spanish *S. aureus* collected from raw meat mixture and fermented sausages over four months belonged to three different clades separated by ≥16,142 SNPs. Likewise, ST398 and ST121 respectively found in Italian soft cheese and raw materials differed for more that 40,315 SNPs. On the other hand, same STs were carried by strains isolated from different countries or contaminating both meat and dairy productions, which clustered together in the phylogenetic tree. The former was the case of ST15, found in Spanish and Moroccan strains from meat productions harboring between 347 and 348 SNPs differences, whereas the latter was ST8 from Italian cheese and Spanish raw meat mixture which differed for 701 SNPs.

Thus, together phylogenetic tree clustering based on SNPs and STs identifications suggested that several *S. aureus* genotypes disseminated through the meat and cheese artisanal plants over time and may not have been linked to the same contamination source. Indeed, ST5, ST8, ST15, ST121 and ST398 have been described in several ecological niches, including humans, food and wildlife (Ghebremedhin et al., 2009; Heaton et al., 2020; Lv et al., 2021; Velasco et al., 2015).

AMR prediction (Table 1) pointed out that all strains presented no antimicrobial resistance determinant genes including *mec* genes encoding from methicillin resistance, one of the resistance traits mostly implicated in *S. aureus* hospital infections worldwide (Wielders et al., 2002). Nevertheless, *blaZ* gene associated to beta-lactams resistance was carried by nine strains including Spanish sausage (ST15 and strain ArFCSA03 with novel ST) and cheese (ST121 and ST398), Italian salami (ST5) and cheese (ST8) as well as Moroccan sausage ST15. Among them, seven strains

exhibited additional AMR genes, such as *tet(K)* and *tet(M)* (tetracycline resistance) in ST15, ST398 and strain ArFCSA03, and *str* (streptomycin resistance) in ST121.

Regarding virulence, all fifteen *S. aureus* genomes carried from 54 to 67 virulence genes (Figure 6). ST121, previously described as ST gathering hypervirulent strains, was associated to genomes carrying the *lukS–lukF* genes associated to the assembly of PVL, which is a bicomponent pore-forming cytotoxin closely related to the development of *S. aureus* infection (Hu et al., 2015). The other thirteen genomes but one (ArFDSA04) carried the *lukF* gene but not the *lukS* gene.

Additionally, ST121, along with one ST15 (ArFMSA01) genome also carried Enterotoxin related gene *seb* (Rao et al., 2015). Other haemolysin related genes were found in all genomes, including *hlb, hld, hlgA,hlgBx, hlgC*.

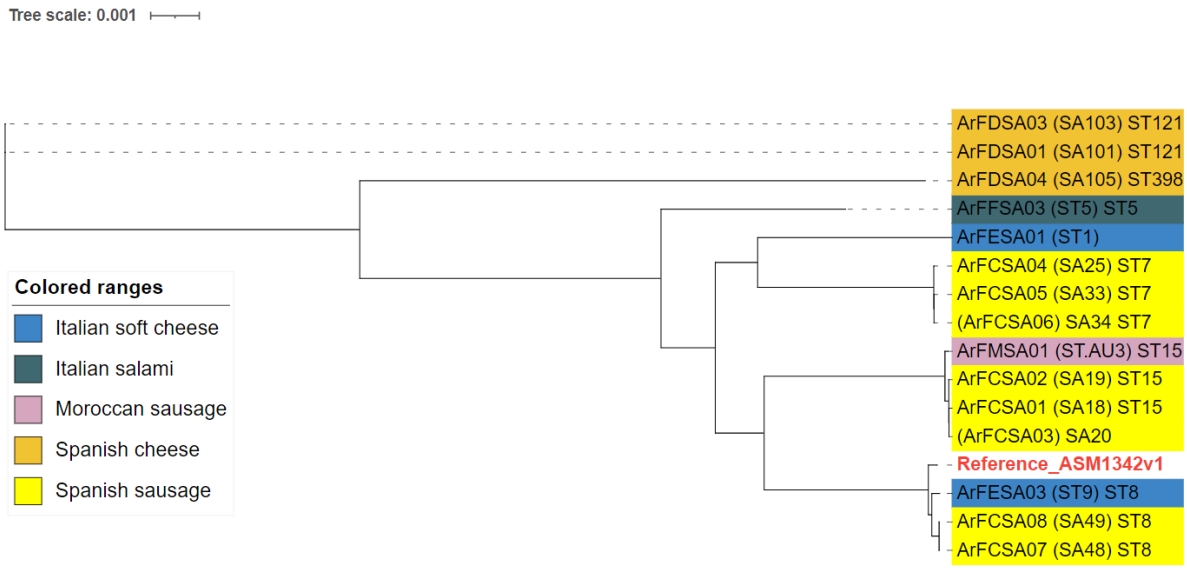

Figure 5: Core-SNPs phylogenetic tree of 15 *S. aureus* strains isolates in cheese and fermented meat productions from Spain, Italy and Morocco.

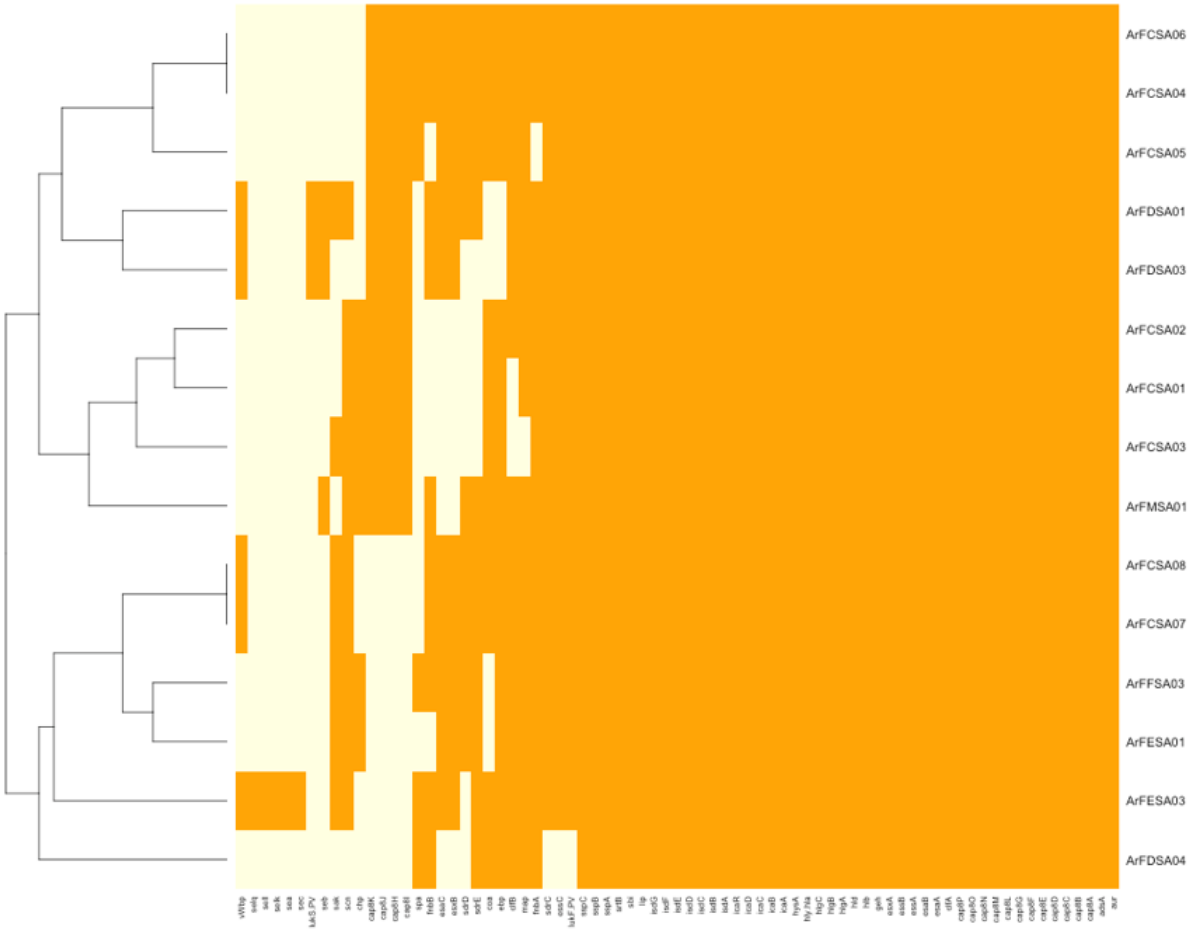

Figure 6: Heatmap showing virulence genes carried by 15 *S. aureus* genomes. Yellow: absence (<80% of sequence identity), orange: presence (>80% of sequence identity).

Conclusions

Overall, WGS-based analyses were effective in building a high-resolution phylogeny among the genomes as well as clarifying their resistome and virulome.

STs distribution supported by SNPs phylogeny suggested that distant related isolates of *L. monocytogenes, S. enterica* and *S. aureus* circulated in the same artisanal production plant over several months contaminating the processing environments and raw materials and then persisting in final products.

Besides AMR intrinsic resistances, multiresistant *S. enterica* was found in Moroccan fermented sausage. Moreover, several virulent or hypervirulent *L. monocytogenes, S. enterica* and *S. aureus* disseminated through the artisanal facilities, suggesting the need of specific attention on control measures able to reduce the risk of these biological hazards in artisanal food productions.

Supplementary material

Sample code	Species	Number of contigs	Total length	Largest contig	GC (%)	N50	N75	L50	L75
ArFCLM01	*L. monocytogenes*	31	2968902	744482	37.95	476695	253636	3	5
ArFCLM02	*L. monocytogenes*	30	2973706	1222483	37.96	516329	253636	2	4
ArFCLM03	*L. monocytogenes*	28	2914813	1203972	37.91	520718	264607	2	4
ArFCLM04	*L. monocytogenes*	28	3007255	587682	37.82	438637	215721	3	6
ArFCLM05	*L. monocytogenes*	29	2799091	754951	37.88	434699	228550	3	5
ArFCLM06	*L. monocytogenes*	31	2915390	1203972	37.91	520718	264607	2	4
ArFCLM07	*L. monocytogenes*	32	2951950	505969	37.84	408574	215721	4	6
ArFCLM08	*L. monocytogenes*	29	2932553	1203972	37.91	533374	264607	2	4
ArFCLM09	*L. monocytogenes*	25	3006740	587682	37.82	438643	215721	3	6
ArFCLM10	*L. monocytogenes*	28	3006828	587683	37.82	438901	215721	3	6
ArFFLM01	*L. monocytogenes*	26	2866457	745246	37.94	475136	253175	3	5
ArFFLM02	*L. monocytogenes*	25	2860941	745246	37.93	475183	253001	3	5
ArFFLM03	*L. monocytogenes*	25	2860650	745246	37.92	475183	252911	3	5
ArFFLM04	*L. monocytogenes*	25	2860779	745246	37.93	475183	253043	3	5
ArFASE02	*S.* Paratyphi B	77	4871281	551422	52.13	319689	133461	6	13
ArFASE03	*S.* Paratyphi B	79	4871099	551424	52.13	319689	145402	6	13
ArFASE04	*S.* Paratyphi B	75	4871712	644777	52.13	231038	145402	6	12
ArFASE05	*S.* Paratyphi B	76	4870999	551383	52.13	364216	145402	6	12
ArFASE06	*S.* Paratyphi B	73	4871497	644777	52.13	399502	145402	5	11
ArFASE07	*S.* Paratyphi B	76	4870999	551385	52.13	364216	145402	6	12
ArFASE11	*S.* Paratyphi B	76	4871003	551422	52.13	364216	145402	6	12
ArFMSE01	*S.* Hadar	46	4673259	735081	52.22	356366	262576	5	8
ArFMSE02	*S.* Kentucky	43	4669097	728786	52.13	518238	322975	4	7
ArFMSE03	*S.* Montevideo	44	4705824	1134579	52.24	614135	312808	3	6
ArFMSE04	*S.* Hadar	53	4703834	1308384	52.23	613464	262534	3	6
ArFMSE05	*S.* Albany	62	4636149	521807	52.18	323876	192517	6	10
ArFMSE06	*S.* Seftemberg	54	4914160	1661467	52.09	557385	237646	3	6
ArFCSA01	*S. aureus*	35	2664558	564837	32.68	306107	151399	3	6
ArFCSA02	*S. aureus*	38	2718693	406882	32.69	289679	160598	4	7
ArFCSA03	*S. aureus*	47	2758691	556881	32.70	306107	87424	3	7
ArFCSA04	*S. aureus*	31	2670475	539965	32.71	397333	315969	3	5
ArFCSA05	*S. aureus*	38	2669035	556767	32.72	315969	248098	4	6
ArFCSA06	*S. aureus*	24	2671095	873106	32.72	603808	397288	2	4
ArFCSA07	*S. aureus*	39	2731332	865930	32.69	815868	172601	2	4
ArFCSA08	*S. aureus*	39	2729924	851790	32.69	815867	172601	2	4
ArFDSA01	*S. aureus*	43	2743255	432694	32.70	181824	93410	5	10
ArFDSA03	*S. aureus*	49	2700499	432694	32.69	230321	70196	4	10
ArFDSA04	*S. aureus*	36	2739656	609892	32.80	339956	151591	3	6
ArFESA01	*S. aureus*	32	2738019	1010428	32.71	493801	127524	2	5
ArFESA03	*S. aureus*	40	2752746	889547	32.65	304438	175520	3	5
ArFFSA03	*S. aureus*	29	2780891	1017875	32.77	469582	167730	2	5

ArFMSA01	*S. aureus*	39	2689124	372180	32.66	243624	158919	5	8

Supplementary Table 1: Assembly statistics of 42 genomes from artisanal food productions of animal origin.

snp-dists 0.6	ArFCLM09	ArFCLM10	ArFCLM01	ArFCLM02	ArFCLM03	ArFCLM04	ArFCLM05	ArFCLM06	ArFCLM07	ArFCLM08	ArFFLM01	ArFFLM02	ArFFLM03	ArFFLM04	Reference
ArFCLM09	0	644	114127	114126	114100	732	23763	114096	599	114095	114009	114022	114024	114029	114127
ArFCLM10	644	0	114067	114066	114040	726	23844	114040	625	114051	113961	113962	113966	113977	114067
ArFCLM01	114127	114067	0	5	9140	114018	113106	9144	114146	9157	8619	8591	8602	8584	0
ArFCLM02	114126	114066	5	0	9139	114017	113105	9143	114145	9156	8618	8590	8601	8583	5
ArFCLM03	114100	114040	9140	9139	0	113996	113170	65	114109	73	9045	9029	9050	9036	9140
ArFCLM04	732	726	114018	114017	113996	0	23858	113999	739	114002	113896	113910	113909	113918	114018
ArFCLM05	23763	23844	113106	113105	113170	23858	0	113161	23785	113160	112937	112955	112953	112959	113106
ArFCLM06	114096	114040	9144	9143	65	113999	113161	0	114109	68	9035	9025	9034	9032	9144
ArFCLM07	599	625	114146	114145	114109	739	23785	114109	0	114110	114022	114031	114035	114043	114146
ArFCLM08	114095	114051	9157	9156	73	114002	113160	68	114110	0	9032	9038	9049	9029	9157
ArFFLM01	114009	113961	8619	8618	9045	113896	112937	9035	114022	9032	0	62	51	65	8619
ArFFLM02	114022	113962	8591	8590	9029	113910	112955	9025	114031	9038	62	0	55	79	8591
ArFFLM03	114024	113966	8602	8601	9050	113909	112953	9034	114035	9049	51	55	0	58	8602
ArFFLM04	114029	113977	8584	8583	9036	113918	112959	9032	114043	9029	65	79	58	0	8584
Reference	114127	114067	0	5	9140	114018	113106	9144	114146	9157	8619	8591	8602	8584	0

Supplementary Table 2 – SNPs distance matrix of *L. monocytogenes* genomes.

snp-dists 0.6.3	Reference	ArFASE02	ArFASE03	ArFASE04	ArFASE05	ArFASE06	ArFASE07	ArFASE11	ArFMSE02	ArFMSE01	ArFMSE04	ArFMSE05	ArFMSE03	ArFMSE06
Reference	0	0	0	0	0	1	0	0	31373	26328	26387	32591	37737	32700
ArFASE02	0	0	0	0	0	1	0	0	31373	26328	26387	32591	37737	32700
ArFASE03	0	0	0	0	0	1	0	0	31373	26328	26387	32591	37737	32700
ArFASE04	0	0	0	0	0	1	0	0	31373	26328	26387	32591	37737	32700
ArFASE05	0	0	0	0	0	1	0	0	31373	26328	26387	32591	37737	32700
ArFASE06	1	1	1	1	1	0	1	1	31374	26329	26388	32592	37738	32701
ArFASE07	0	0	0	0	0	1	0	0	31373	26328	26387	32591	37737	32700
ArFASE11	0	0	0	0	0	1	0	0	31373	26328	26387	32591	37737	32700
ArFMSE02	31373	31373	31373	31373	31373	31374	31373	31373	0	31196	31193	27336	36308	28344
ArFMSE01	26328	26328	26328	26328	26328	26329	26328	26328	31196	0	411	32233	38427	32577
ArFMSE04	26387	26387	26387	26387	26387	26388	26387	26387	31193	411	0	32211	38405	32555
ArFMSE05	32591	32591	32591	32591	32591	32592	32591	32591	27336	32233	32211	0	35678	26926
ArFMSE03	37737	37737	37737	37737	37737	37738	37737	37737	36308	38427	38405	35678	0	35125
ArFMSE06	32700	32700	32700	32700	32700	32701	32700	32700	28344	32577	32555	26926	35125	0

Supplementary Table 3 – SNPs distance matrix of *S. enterica* genomes.

snp-dists 0.6	Reference	ArFDSA01	ArFDSA03	ArFDSA04	ArFCSA01	ArFCSA02	ArFCSA03	ArFCSA04	ArFCSA05	ArFCSA06	ArFMSA01	ArFCSA07	ArFCSA08	ArFESA01	ArFFSA03	ArFESA03
Reference	0	39067	38693	47105	16230	17163	17348	17550	17523	17461	15979	903	910	18885	19880	963
ArFDSA01	39067	0	40	40344	36982	37132	37357	37076	37028	36979	36880	38801	38825	37374	37650	38749
ArFDSA03	38693	40	0	40315	36888	37018	37076	36957	36909	36859	36811	38457	38469	37069	37310	38468
ArFDSA04	47105	40344	40315	0	44900	45168	45206	45032	45013	44986	44847	45895	45897	46007	45576	45935
ArFCSA01	16230	36982	36888	44900	0	36	28	17099	17100	17070	348	16142	16142	17468	18041	16189
ArFCSA02	17163	37132	37018	45168	36	0	34	17176	17176	17159	347	16386	16381	17627	18212	16409
ArFCSA03	17348	37357	37076	45206	28	34	0	17227	17223	17193	342	16405	16403	17848	18364	16623
ArFCSA04	17550	37076	36957	45032	17099	17176	17227	0	17	23	16970	17506	17514	17359	18269	17578
ArFCSA05	17523	37028	36909	45013	17100	17176	17223	17	0	10	16976	17492	17501	17364	18247	17554
ArFCSA06	17461	36979	36859	44986	17070	17159	17193	23	10	0	16949	17428	17434	17315	18227	17501
ArFMSA01	15979	36880	36811	44847	348	347	342	16970	16976	16949	0	15974	15972	17374	17958	16009
ArFCSA07	903	38801	38457	45895	16142	16386	16405	17506	17492	17428	15974	0	1	18099	19052	701
ArFCSA08	910	38825	38469	45897	16142	16381	16403	17514	17501	17434	15972	1	0	18118	19062	701
ArFESA01	18885	37374	37069	46007	17468	17627	17848	17359	17364	17315	17374	18099	18118	0	19890	18215
ArFFSA03	19880	37650	37310	45576	18041	18212	18364	18269	18247	18227	17958	19052	19062	19890	0	19106
ArFESA03	963	38749	38468	45935	16189	16409	16623	17578	17554	17501	16009	701	701	18215	19106	0

Supplementary Table 4 – SNPs distance matrix of *S. aureus* genomes.

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Title: Resistome and virulome diversity of foodborne pathogens isolated from artisanal food production chain of animal origin in the Mediterranean region

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Study 3

Genomic features of Klebsiella isolates from artisanal ready-to-eat food production facilities

Abstract

Increasing reports on *K. pneumoniae* strains with antimicrobial resistance and virulence traits from food and farm animals are raising concerns about the potential role of *Klebsiella* spp. as a foodborne pathogen. This study aimed to report and characterize *Klebsiella* spp. isolates from two artisanal ready-to-eat food (soft cheese and salami) producing facilities, and to track similar genotypes in different ecological niches.

Over 1,170 samples were collected during the whole production chain of different food batches. The overall *Klebsiella* prevalence was 6%. Strains were classified into the three *Klebsiella* species complexes: *K. pneumoniae* (KpSC, n=17), *K. oxytoca* (KoSC, n=38) and *K. planticola* (KplaSC, n=18). Despite high genetic diversity we found in terms of known and new sequence types (STs), core genome phylogeny revealed clonal strains persisting in the same processing setting for over 14 months, isolated from the environment, raw materials and end-products.

Strains showed a natural antimicrobial resistance phenotype-genotype. *K. pneumoniae* strains showed the highest virulence potential, with sequence types ST4242 and ST107 strains carrying yersiniabactin *ybt16* and aerobactin *iuc3*. The latter was detected in all *K. pneumoniae* from salami and was located on a large conjugative plasmid highly similar (97% identity) to *iuc*3^{+} plasmids from human and pig strains circulating in nearby regions of Italy.

While identical genotypes may persist along the whole food production process, different genotypes from distinct sources in the same facility shared an *iuc*3-plasmid. Surveillance in the food chain will be crucial to obtain a more comprehensive picture of the circulation of *Klebsiella* strains with pathogenic potential.

Introduction

The *Klebsiella* genus belongs to the *Enterobacteriaceae* family and comprises capsulated Gram-negative, rod-shaped, facultative anaerobic bacteria, which are ubiquitously found in a wide range of host-associated and environmental niches, including soil, surface waters, plants and gastrointestinal tracts of animals and humans (Brisse et al., 2006; Klaper et al., 2021). *Klebsiella* encompasses several species including pathogenic strains of important public health concern in nosocomial settings (e.g., *K. pneumoniae*). The *Klebsiella pneumoniae* species complex (KpSC) currently includes seven major phylogroups: *K. pneumoniae* sensu stricto (Kp1), *K. quasipneumoniae* subsp. *quasipneumoniae* (Kp2), *K. variicola* subsp. *variicola* (Kp3), *K. quasipneumoniae* subsp. *similipneumoniae* (Kp4), *K. variicola* subsp. *tropica* (Kp5), '*K. quasivariicola'* (Kp6) and *K. africana* (Kp7) (Blin et al., 2017; Brisse & Verhoef, 2001; Fevre et al., 2005; Long et al., 2017; Rodrigues et al., 2019; Wyres et al., 2020). Similarly, the *Klebsiella oxytoca* species complex (KoSC) comprises nine phylogroups, from Ko1 to Ko9, defined in accordance to the chromosomal variants belonging to the classe A beta-lactamase (*bla*$_{OXY}$) gene, with *K. michiganensis* (Ko1) including the Ko5 sublineage (Fevre et al., 2005), *K. oxytoca* sensu stricto (Ko2), *K. spallanzanii* (Ko3) embracing the sublineage Ko9 (Merla et al., 2019), *K. pasteurii* (Ko4), *K. grimontii* (Ko6), *K. huaxensis* (Ko8) and the undetermined taxonomic status of Ko7 (Izdebski et al., 2015; Yang et al., 2021). *Klebsiella planticola* was firstly described in 1981 (Bagley et al., 1981), whereas *K. ornithinolytica* was introduced 8 years later (Sakazaki et al., 1989). In 2001, based on the molecular analysis of 16S ribosomal RNA (rRNA) and RNA polymerase β subunit encoding genes (*rpoB*), *K. planticola* and *K. ornithinolytica* were classified into a new genus, named as *Raoultella* (Drancourt et al., 2001). Thirteen years later, a new species *Raoultella electrica* was discovered (Kimura et al., 2014). A reunification of *Raoultella* and *Klebsiella* into the single genus *Klebsiella* has subsequently been proposed, since phylogenomic analyses showed that the genus *Raoultella* is nested within *Klebsiella*, thus not supporting the assignment of *Raoultella* species as a separate genus. According to this reclassification, the *Klebsiella planticola* species complex (KplaSC) encompasses four phylogroups: *K. planticola* (Kplan1), *K. ornithinolytica* (Kplan2), *K. electrica* (Kplan3) and Kplan4 representing an undescribed species (Ma et al., 2021).

K. pneumoniae and to a lesser degree *K. oxytoca,* are associated to hospital-acquired and community-onset infections such as bacteremia, pneumonia, meningitis, and urinary tract infections, which are particularly worrisome in immunocompromised individuals (Gómez et al., 2021; Lin et al., 2010; Podschun & Ullmann, 1998). One of the major concerns with *Klebsiella* infections, especially with *K. pneumoniae*, is the emergence and dissemination of isolates producing extended-spectrum β-lactamases (ESBL) and/or carbapenemases, and showing multi-drug resistance (MDR), which impairs the clinical management of healthcare-associated infections (Fair & Tor, 2014). This has led to the declaration of *K. pneumoniae* as a critical priority pathogen by the World Health Organization (WHO) (Tacconelli et al., 2018). Besides nosocomial bacteremia, invasive community-acquired infections are mostly linked to so-called hypervirulent *K. pneumoniae* strains (hvKp). Initially described in Asian countries, hvKp isolates commonly harbor horizontally-acquired virulence factors

encoding for siderophores systems such as yersiniabactin (Ybt), aerobactin (Iuc/iut) and salmochelin (Iro) (Lam et al., 2018).

Beyond clinical settings, *Klebsiella* spp. have been also found in several food products. Whilst not typically considered as foodborne pathogens, *Klebsiella* spp. isolates in food deserve further investigation for several reasons (Hartantyo et al., 2020). First, *Klebsiella* spp. ability in colonizing the gastrointestinal tract after food consumption often precedes infection (Martin et al., 2016; Wyres & Holt, 2018). Second, antimicrobial-resistance (AMR) or virulence genes, mostly present in mobile genetic elements, could be transferred to other pathogens found in the same ecosystem, leading to the emergence of new resistance or pathogenesis mechanisms. In this regard, some studies pointed out that *K. pneumoniae* could potentially act as reservoir of AMR genes in the food chain, colonizing poultry and meat products (Davis et al., 2015; Guo et al., 2016; Kim et al., 2005), and/or of genetic elements associated with enhanced virulence (K1, K2, and K54 capsular serotypes and *wcaG*) in raw or ready-to-eat (RTE) foods (Hartantyo et al., 2020).

At present, knowledges on the prevalence rates of antibiotic resistant and/or virulent *Klebsiella* spp. isolates from food chains is limited. In particular, artisanal productions are widely appreciated among consumers and, in Italy, contribute to the national cultural heritage. While for some products the authenticity of traditional production methods is protected by European legal designations (eg., PDO, PGI and STG), others result in lack of production standardization. Not-standardized productive processes combined with missing extensive automation in small-scale facilities could pose severe hazards in terms of final product's microbial safety, especially when products result from short fermentations and/or are consumed without cooking. Examples of safety issues associated to artisanal fermentative processes are represented by biological (foodborne pathogens) or chemical (mycotoxins or biogenic amines) contaminants generated in the context of spontaneous fermentations, which could represent a severe hazard for human health (Capozzi et al., 2020).

Given the above, we aimed to investigate *Klebsiella* spp. isolated from the artisanal food chain to gather evidence on the public health risk that artisanal RTE foods may pose as vehicles of *Klebsiella* strains. We focused our study on *Klebsiella* strains (broadly comprising its different species complexes *K. pneumoniae*, *K. oxytoca* and *K. planticola*) repeatedly isolated from two Italian artisanal RTE food productions (salami and cheese) over a sampling period covering six commercial batches. We (i) assessed the occurrence of *Klebsiella* spp. strains through the selected food productions and performed genomic sequencing to (ii) improve their taxonomic characterization and (iii) describe the circulating genotypes and their genetic features of proven clinical importance (i.e., antimicrobial resistance and virulence). Finally (iv) we compared our sequences with public databases to evaluate the genetic relatedness between our food isolates and previous ones from other ecological niches.

Materials and methods

Sampling procedure

In this study, 1,170 samples were collected between January 2020 and May 2021 from six batches of dairy and pork meat-based productions in two artisanal RTE small-scale factories located in the Northern Italy. The dairy production corresponded to a soft cheese prepared with pasteurized cow milk (Pasquali et al., 2022). The other production was represented by a fermented dried sausage, so-called "salami", prepared using pork meat from an Italian swine autochthonous breed with no addition of starter cultures or nitrites/nitrates. Within both artisanal facilities, the six commercial batches were produced in different months, to reflect a whole seasonality cycle, but respecting the same traditional methods from raw materials to the end of the production line (cheese and salami end-products). The sampling included raw materials and final products collected at each key processing stage, plus environmental swabs picked up from surfaces, machines, and operator's gloves, while the food was managed within the processing rooms (see Supplementary Figure 1 in Study 1). The latter were collected before cleaning and disinfection procedures, by swabbing a 100 cm2 area with a sterile cotton swab (Copan Italia, Brescia, Italy) that had been moistened in 10 mL of saline solution (0.9% NaCl). A total of 420 and 750 samples were collected from salami and cheese productions, respectively (Table 1).

Artisanal RTE food production	**Sample origin**	**Description**	**Number (n)**
Salami	Environmental	Wall inside stuffing, drying and ripening area	90
		Manhole inside stuffing, drying and ripening area	90
		Processing surfaces inside stuffing area	30
		Filler stuffer machine located within the stuffing area	30
	Raw materials	Minced meat mixture managed within the stuffing area	30
		Salami stored for 1 week inside drying area	30
		Salami stored for 3 weeks inside ripening area	30
		Salami stored for 10 weeks inside ripening area	30
		Salami stored for 18 weeks inside ripening area	30
	Final product	Ripened salami (stored for 28 weeks inside ripening area)	30
Total			**420**
Cheese	Environmental	Wall inside warm, maturation and packaging area	90
		Manhole inside warm, maturation and packaging area	90
		Gloves of workers inside the packaging area	30
	Raw materials	Raw and pasteurized milk, calf rennet and raw cheese stored within warm and maturation room	150
	Final product	Cheese packed on the same day ("day 0") of production along with stored cheese at day 4, 8, 11 and 15 at 2°C, 8°C and 2°C for 5 days followed by 8°C for 10 days.	390
Total			**750**

Table 1: Overview of the 1,170 samples collected across six batches of salami and cheese production in the artisanal facilities.

Isolation of Klebsiella spp.

For both cheese and meat productions, one aliquot of 25g from samples of raw materials as well as intermediate and final products along with swabs picked up from the processing environment were enriched in buffered peptone water (Thermo Scientific™) at 37 °C overnight. A 10-µL loopful of the overnight-enriched culture was streaked onto MacConkey agar (Thermo Scientific™) and incubated again at 37°C for 24 h. Colonies showing the typical pink mucoid morphology related to *Klebsiella* spp. were picked and submitted to biochemical test (RapID™ ONE System, Thermo Scientific™) for preliminary species confirmation. DNA extraction (Chelex method, Kimura et al., 1999) was then performed on strains identified as *K. pneumoniae* and *K. oxytoca* for species identification by multiplex polymerase chain reaction (Chander et al., 2011) (multiplex-PCR). *Klebsiella* spp. positive isolates (n=75) were stored at −80°C in Brain Heart Infusion broth (BHI, Thermo Scientific™) with 20% glycerol until further use.

Antimicrobial susceptibility testing

After species confirmation, antibiotic susceptibility testing of *Klebsiella* spp. strains was carried out using the Sensititre™ EUVSEC ready to use plates (Thermo Scientific, USA) and following the gold standard broth microdilution phenotypic assay (ISO, 2019). Following the manufacturer's protocol, isolates were tested against 13 antibiotics: trimethoprim (TMP), ciprofloxacin (CIP), tetracycline (TET), meropenem (MERO), azithromycin (AZI), nalidixic acid (NAL), cefotaxime (FOT), chloramphenicol (CHL), tigecycline (TGC), ceftazidime (TAZ), colistin (COL), ampicillin (AMP) and gentamicin (GEN). The isolates were defined as susceptible or resistant according to the clinical breakpoints (CBP) established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), except for those antimicrobials for which a CBP was not available (EUCAST, 2020). In particular, CBPs of the Clinical and Laboratory Standard Institute (CLSI) were applied for tetracycline, azithromycin and nalidixic acid (Weinstein et al., 2021).

DNA extraction and whole genome sequencing (WGS)

Klebsiella spp. isolates confirmed by biochemical test and PCR were grown overnight at 37°C on BHI broth (Thermo Scientific™). DNA extraction for WGS purposes was performed with the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. BioSpectrometer fluorescence (Eppendorf) was used to measure the purified DNA concentration and the quality parameter ratio 260/280. Libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, Milan, Italy) and the whole genome of selected isolates was paired end sequenced (250 bp) using the MiSeq platform (Illumina). Reads quality assessment was carried out with Kraken2 v2.1.1 (https://github.com/DerrickWood/kraken2). Raw reads with good quality were pre-processed and *de novo* assembled using fq2dna v21.06 (https://gitlab.pasteur.fr/GIPhy/fq2dna), which also performed the post-processing scaffold sequence accuracy assessment. Contiguity metrics was plotted with ggplot2 package in R v4.1.2 (https://cran.r-project.org/).

Genome-based taxonomic assignment and sequence typing

The taxonomy at species levels, previously assigned with biochemical and molecular testing, was then assessed by WGS using different tools: Kraken2 v2.1.1 (with the pre-buit MiniKraken DB) (https://github.com/DerrickWood/kraken2) for taxonomic classification of reads, ReferenceSeeker v1.7.3 for average nucleotide identity (ANI), based search of closely related reference genomes from RefSeq (https://www.ncbi.nlm.nih.gov/refseq), and Kleborate v2.0.0 (https://github.com/katholt/Kleborate) for rapid and accurate species and subspecies prediction based on Mash distances calculated against a taxonomically curated genome set of *Klebsiella* spp. as well as other Enterobacterales assemblies. Results from the three different tools were combined to provide a more accurate species assignment. For genomes with discordant results (n=7), hits from Kleborate were retained due to the high specificity of this tool for species and subspecies identification for *K. pneumoniae* and its associated species complexes.

Known sequence types (STs) were assigned using Kleborate and MLST v2.19.0 (https://github.com/tseemann/mlst) based on the multilocus sequence typing (MLST) schemes hosted in the BIGSdb-Pasteur (https://bigsdb.pasteur.fr/klebsiella/) and pubMLST (Jolley et al., 2018). *K. pneumoniae* and *K. oxytoca* isolates with unknown alleles and sequence types were submitted to BIGSdb-Pasteur.fr and pubMLST.org platforms, respectively, for the definition of novel alleles and STs.

Antimicrobial resistance and virulence genes detection, and location in the genomic context

The software Kleborate was used to screen the *Klebsiella* spp. genome assemblies against a curated database of key virulence and AMR loci using a minimum threshold of 90% for nucleotide identity and gene coverage (Lam et al., 2021). New variants of the aerobactin and yersiniabactin sequence types found in *K. pneumoniae* isolates were submitted to BIGSdb-Pasteur.fr for definition of the novel AbST and YbST. Plasmid sequences were reconstructed from genome assemblies using the MOB-recon tool of MOB-suite v3.0.1 (https://github.com/phac-nml/mob-suite) (Robertson & Nash, 2018). The typing option was also enabled to predict plasmids' mobility. Moreover, a plasmid multilocus sequence typing (pMLST) was performed on the subset of *K. pneumoniae* draft genomes using the pMLST Web tool (http://cge.cbs.dtu.dk/services/pMLST/) selecting the IncF RST configuration.

Comparison of virulence-related plasmids with public data

To draw a broader picture of the possible ecological niches in which virulence-related plasmids similar to those herein detected can circulate, a pairwise comparison of plasmids from this study and selected sequences from public databases (e.g., Microreact and NCBI) was performed with fastANI v1.33 (https://github.com/ParBLiSS/FastANI). The sequences of virulence-related plasmids were also annotated with PROKKA v1.14.5 (https://github.com/tseemann/prokka) (Seemann, 2014) and a manual curation of hypothetical proteins was performed searching for homologous protein in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Annotated proteins were then submitted to Clinker v0.0.23 (https://github.com/gamcil/clinker) for interactive visualization of protein clusters and alignments.

Pan-genome calculation

The entire gene repertoire of *Klebsiella* strains from the artisanal productions was assessed reconstructing the pangenome with Panaroo v1.2.3 (https://github.com/gtonkinhill/panaroo), selecting the sensitive mode with 95% of sequence identity and core-gene thresholds (Tonkin-Hill et al., 2020). The resulted alignment was further used to build a Maximum-likelihood (ML) phylogenetic tree with IQ-Tree using the substitution model GTR+F+R8 in v2.0.6 (https://github.com/Cibiv/IQ-TREE) (Kalyaanamoorthy et al., 2017; Nguyen et al., 2015). The distribution of core and accessory genes was observed across the phylogenomic tree and the samples' metadata on the Phandango website (https://jameshadfield.github.io/phandango/#/) for interactive pangenome visualization.

Gene-by-gene approach and phylogenetic analyses

To provide insight into strains relatedness, the ML tree inferred on core genome multiple sequence alignments was visualized with ITOL (https://itol.embl.de/) along with samples metadata (e.g., food product, batch, and sample origin), STs and virulence information. For KplaSC, as no MLST schemes has been defined yet, arbitrary identifiers (from new1 to new6) were attributed to the different strains based on the core gene phylogeny branches.

A core genome MLST (cgMLST) approach was then used to assess the genetic distance of genotypes from the artisanal production within an extended context of public strains selected from the National Centre for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) and BIGSdb-Pasteur (https://bigsdb.pasteur.fr/klebsiella). The *K. pneumoniae* strains were uploaded on the BIGSdb-*Kp* database and compared with the *K. pneumoniae* strains belonging to the same ST (n=15) using the 629 loci cgMLST scheme, scgMLST629_S, hosted and curated on the platform (Hennart et al., 2022). There was no cgMLST scheme available to compare KoSC and KplaSC genotypes. We therefore used the chewBBACA suite v2.8.5 (https://github.com/B-UMMI/chewBBACA) and built dedicated cgMLST schemes to separately analyze KoSC and KplaSC strains. For scheme creation, all public genomes were firstly downloaded from the NCBI genome repository, representing the overall population of KplaSC (*K. planticola* and *K. ornithynolitica*) and KoSC (*K. oxytoca*, *K. michiganensis*, *K. grimontii* and *K. pasteurii*) isolated worldwide. Further, eight strains from the Collection of Institut Pasteur (CIP, Paris, France) (n=1 *K. planticola*, n=2 *K. ornithynolitica* for KplaSC; n=1 *K. oxytoca*, n=1 *K. michiganensis*, n=2 *K. grimontii* and n=1 *K. pasteurii* for KoSC), and six *K. oxytoca* reference strains (ATCC® 700324™, ATCC® 13182™, ATCC® 49131™, ATCC® 51983™, ATCC® 13030™ and ATCC® BAA-3059™; retrieved at https://www.atcc.org/) were included in the public dataset. A first screening of public genomes was carried out based on assemblies' quality metrics, by filtering out sequences with more than 500 contigs, total genome size outside the typical length of *Klebsiella* spp. (<4.5 Mb and >6.5 Mb) and N50 lower than 30000. Strains with mixed species as calculated by the ribosomal MLST based species identification tool (https://pubmlst.org/species-id) were also excluded (Jolley et al., 2012). The remaining assemblies were then clustered together with artisanal food genomes based on pairwise MASH-based distance (https://gitlab.pasteur.fr/GIPhy/JolyTree), to carefully select reference genomes for the scheme construction. Representative genomes for each

cluster were selected based on best contiguity metrics, and the diversity of available metadata (location and collection date) and of sequence types (classical MLST or rMLST where MLST was not available). This selection was aimed at embracing the broadest population diversity while preventing redundancy of the dataset. The selected references, 51 and 104 genomes for KplaSC and KoSC, respectively, were used to build a cgMLST scheme for each species complex (see Supplementary Table S1 for public genomes list and metadata from NCBI, downloaded with NCBImeta v0.8.3, https://github.com/ktmeaton/NCBImeta). The cgMLST schemes were built following recommendations and steps available in the chewBBACA guidelines (https://github.com/B-UMMI/chewBBACA#i-whole-genome-multilocus-sequence-typing-wgmlst-schema-creation). Paralogous loci were removed from the scheme and the loci present in at least 99% of samples were extracted. The quality of core loci was estimated with the chewBBACA *Schema Evaluation* module and the reported loci with high allele variability were excluded from the scheme. The cgMLST schemes were finally composed of 3,272 loci for KoSC and 2,957 loci for KplaSC. The schemes were deposited in a Zenodo repository for public access (10.5281/zenodo.7477602). We used these schemes to define the allelic profiles of strains from food production facilities and a collection of public strains from other sources (Supplementary Table S1) and with the lowest genetic distance based on MASH analysis (https://gitlab.pasteur.fr/GIPhy/JolyTree). Pairwise distances between allele profiles were then analyzed using the Minimum Spanning Tree (MST)-based clustering tool MSTclust v0.21b (https://gitlab.pasteur.fr/GIPhy/MSTclust) to identify clusters of strains that share a closely related genotype (e.g., ~1.5% of allele distances across the dedicated cgMLST scheme).

Results

Despite the low prevalence, several Klebsiella spp. contaminate the food processing facilities

Over 1,170 samples were collected across six commercial batches sequentially produced from January 2020 to May 2021 from two artisanal food production chains (cheese and salami). The overall prevalence of the *Klebsiella* spp. strains across both productions was 6%. In total, 75 *Klebsiella* isolates were found and identified as *K. oxytoca* (n=52) and *K. pneumoniae* (n=23) based on biochemical test (RapID™ ONE System, Thermo Scientific™) and multiplex-PCR (Chander et al., 2011) (Supplementary Table S2), followed by whole-genome sequencing (WGS). Using genome-based taxonomic designations, 73 out of 75 isolates were classified in 3 *Klebsiella* species complexes: 17 (23%) KpSC isolates, including 16 *K. pneumoniae* sensu stricto and 1 *K. variicola* subsp. *variicola*; 38 (52%) KoSC of which 11 *K. oxytoca sensu stricto*, 15 *K. michiganensis*, 5 *K. pasteurii* and 7 *K. grimontii*; and 18 (25%) KplaSC with 13 *K. ornithinolytica* and 5 *K. planticola* (Supplementary Table S2, Figure 1). Two isolates previously identified as *Klebsiella* spp. were re-classified as *Citrobacter* spp. and thus excluded from the dataset. In summary, using genomics data, we were able to improve species identification for the 28% of the isolates previously identified by biochemical and molecular testing (Supplementary Table S2).

Klebsiella spp. isolates were detected in all batches of salami and five of six batches of cheese, and were isolated along approximately the whole sampling period (Figure 1, Supplementary Table S2). The species distribution across food productions, batches and sample origin showed a higher recovering of KpSC isolates from salami (94%; n=16/17) and KoSC isolates from cheese (71%; n=27/38). Regarding KplaSC, *K. ornithinolytica* isolates were retrieved from four batches of cheese samples (collected in warm and maturation room as well as stored cheese) and in the associated environment. On the other hand, *K. planticola* detection was restricted to the salami production and represented strains collected from the final product (RTE salami with 28-weeks ripening). Strain's taxonomy assignments, distribution across samples and batches are showed in Figure 1.

Draft genomes generated through *de novo* assembly of Illumina short reads showed good contiguity metrics (Supplementary Fig. S1): low number of contigs (33 – 280 bp) and high N50 (161,990 – 1,140,929); genome length (5.3 - 6.5 Mb); GC contents (54.9% - 57.4%) and number of predicted coding sequences (CDS; 4,900 – 6,193), in the typical range for *Klebsiella* spp. (Supplementary Table S3). The median values of number of contigs, cumulative size, and number of CDSs were higher for the KoSC (85 contigs, 6.08 Mb and 5,640 CDSs) and KplaSC (72 contigs, 5.8 Mb and 5,404 CDSs) compared to KpSC (43 contigs, 5.4 Mb and 5,008 CDSs). On the other side, KpSC presented the highest GC content (~57%) in comparison to ~55% of other *Klebsiella* species complexes.

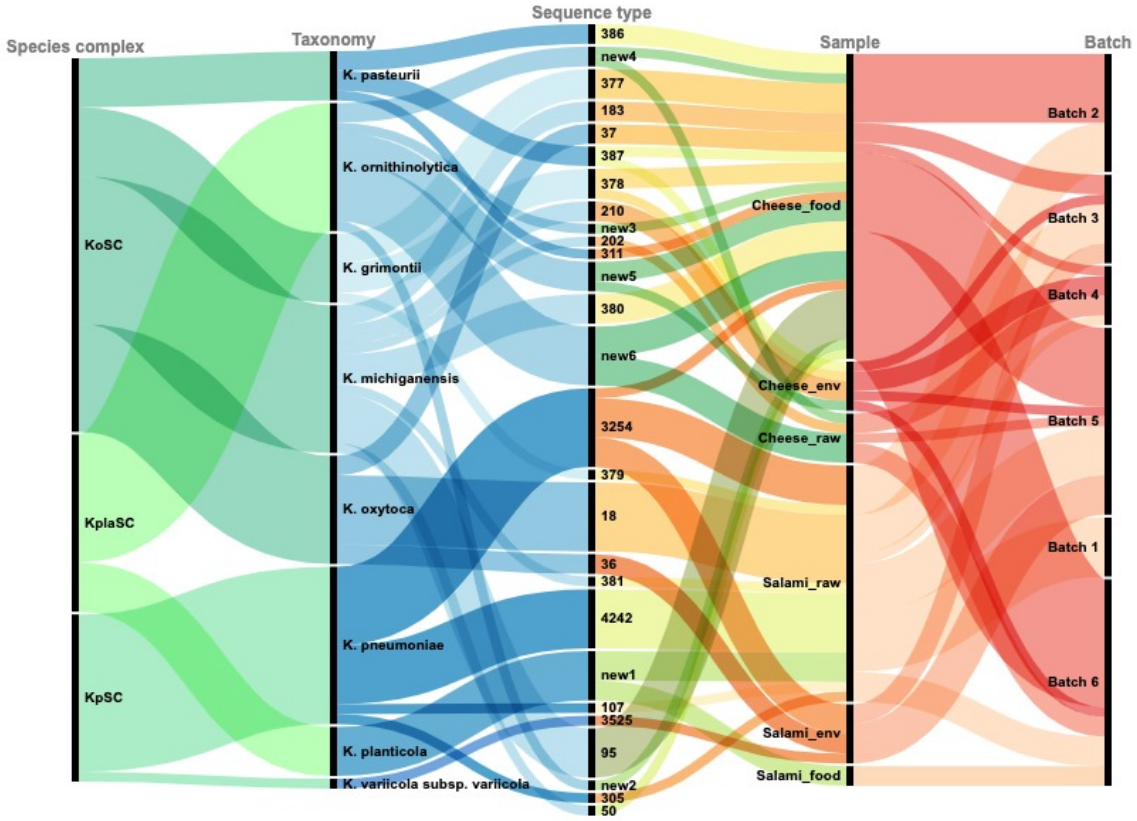


Figure 1: Alluvial diagram showing the distribution of 73 *Klebsiella* spp. strains belonging to KoSC, KplaSC and KpSC complexes among species, tested samples (the environment: env; intermediate: raw and final products: food), batches as well as sequence types. The diagram was created with RAWGraphs 2.0 beta (https://app.rawgraphs.io/).

Most strains show a natural antibiotic susceptibility phenotype

Results from antibiotic susceptibility testing (AST) were obtained for 72 out of 75 isolates (Supplementary Table S4) and 71 showed a natural antibiotic susceptibility phenotype. All isolates except one showed resistance to ampicillin (MIC ≥32), as expected for *Klebsiella* spp. due to the presence of an intrinsic chromosomal type A beta-lactamase. The exception was one *K. ornithinolytica* strain collected from cheese with a MIC=8, which is the upper limit to be considered as susceptible (EUCAST, 2020). Only two isolates (from cheese and salami productions) showed resistance to azithromycin (MIC=32).

Multiple sequence types circulate across both food facilities

We recovered sequence types (STs) for all isolates except KplaSC ones, based on the 7-MLST loci schemes available in BIGSdb-Pasteur for KpSC (https://bigsdb.pasteur.fr/klebsiella/) and PubMLST for KoSC (https://pubmlst.org/organisms/klebsiella-oxytoca) (Supplementary Table S5). Overall, 21 STs were identified for KpSC and KoSC, including 9 novel STs. We identified 4 known and 1 new ST (defined as ST5929) within the KpSC, as well as 8 known and 8 new STs (defined as ST311, ST377, ST378, ST379, ST380, ST381, ST386, ST387) within the KoSC (Supplementary Table S5). The 35% and 41% of KpSC isolates were respectively designed as ST4242 and ST3254. ST3254 was found in two salami batches in raw materials, ripened salami (up to ten weeks) and the environment, suggesting that this strain likely persisted for several months in the processing environment. In contrast, ST4242 was only found in raw material samples from salami but distributed among different batches, suggesting a possible introduction in the food production line from animals raised in livestock farming. A similar distribution was reported for KoSC strains, with isolates sharing the same unique ST even though they came from the different sample's origins (environment and stored cheese) or batches (Figure 1).

Some genotypes persist in the food processing facilities and circulate in other niches

To address the question of strains relatedness and genotypes circulation across artisanal facilities, we inferred a maximum likelihood (ML) phylogenetic tree from the core-genome alignment. As expected, the phylogenomic reconstruction shaped three distinct three major clades according to the *Klebsiella* species complexes (Figure 2). Within each clade, strains clusters and subclusters were displayed according to the species and, more in depth, to the ST, with strains from different STs but harboring similar virulence features grouped into tight branches. For each species complex, clonal groups of isolates (i.e., clonal genotypes - corresponding to the same ST in KpSC and KoSC) were contaminating the processing facilities overtime. Clonal genotypes were detected across different origins (raw materials, food and environment) and all along the whole production of batches, from raw materials to the final products (i.e., the same *K. planticola* genotype was found from minced meat up to 28 weeks ripening salami).

A cgMLST approach was used to investigate clonal relationships of our *Klebsiella* spp. from artisanal food in the context of selected public genomes (n=94) representing the same taxonomic species and

STs. We identified <1.5% of pairwise allele distances (8-9 different alleles) among ST3254 strains sampled from salami (n=8) and human carriage from Africa (n=2), using the 629 loci cgMLST scheme hosted in the BIGSdb Klebsiella database (Hennart et al., 2022) (Supplementary Table S6). We further identified a ~1.5% genetic distance (50 allele variants across a 3,272-loci cgMLST scheme designed in this study) in a cluster of ST37 *K. oxytoca* isolates from the Italian cheese sampled along its shelf-life (n=2) and human carriage and environmental *K. oxytoca* strains isolated in Europe (n=6). No clusters were observed at ≤1.5% pairwise distance for KplaSC isolates from this study and public repositories using the here designed 2,957-loci cgMLST scheme (Supplementary Table S7).

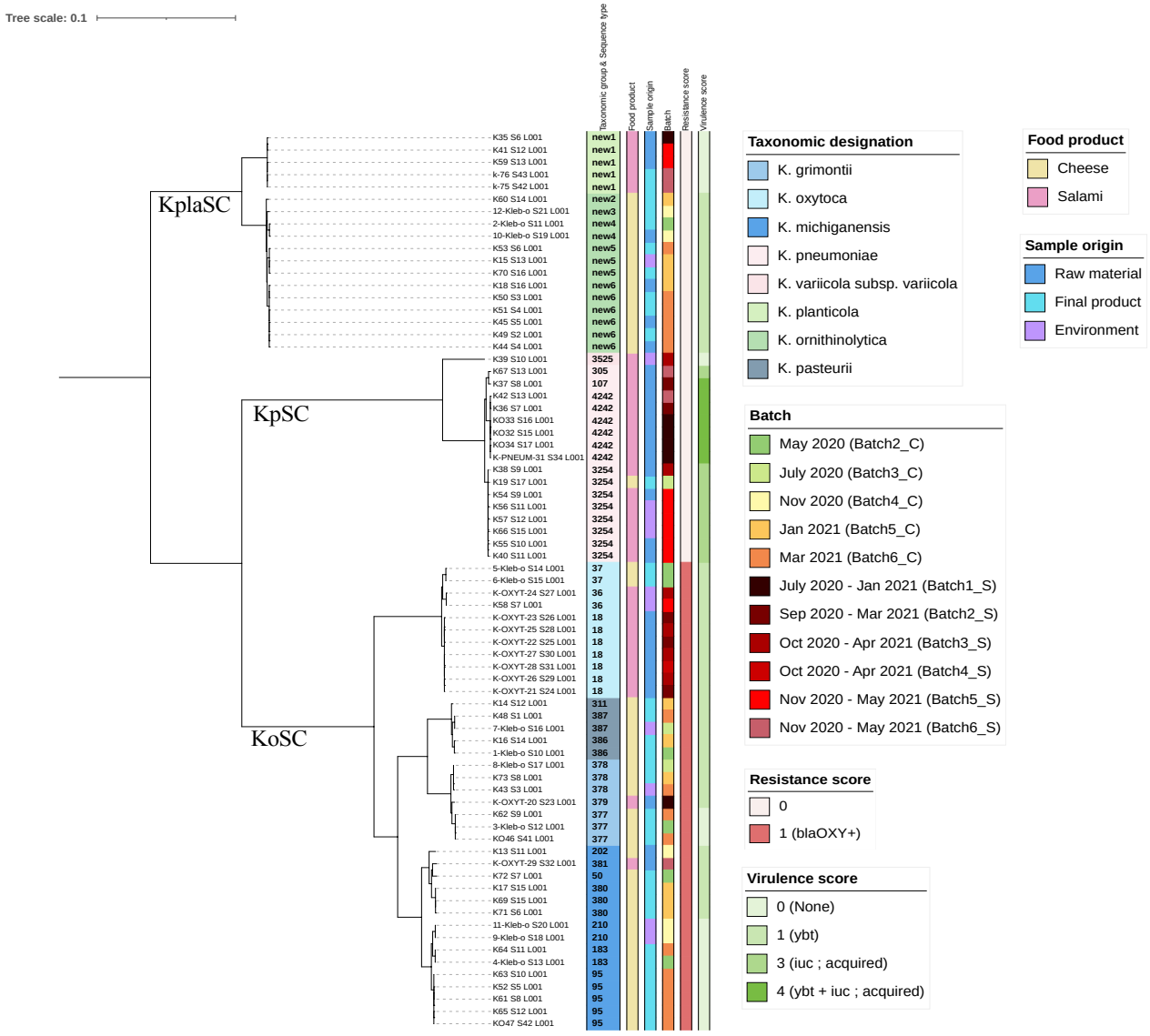


Figure 2**:** Maximum likelihood phylogenetic tree inferred from core gene alignments of 73 *Klebsiella* genomes isolated from two Italian meat and dairy artisanal food productions. Species complexes (Kpla, Kp and Ko) are indicated along the branches. The tree was rooted with the KplaSC. Three main clusters correspond to the species complexes with color highlights in the first columns indicating the ST: KplaSC (green), KpSC (pink) and KoSC (blue). Taxonomic designation as well as sequence type, if available, are

indicated. Sample metadata (food product, sample origin and batch) and resistance and virulence scores given by Kleborate are shown on the figure color key on the right.

Detection, distribution and mobilization of AMR, virulence, and serotype determinants

Virulence and AMR genes detected by Kleborate were summarized in Supplementary Table S8. Overall, few antimicrobial resistance determinants were found as expected from the AST results. KpSC strains carried only the intrinsic β-lactamase-encoding genes *bla*SHV (*K. pneumoniae*) and *bla*LEN (*K. variicola*). KoSC strains from both artisanal productions harbored the β-lactamase-encoding bla_{OXY-1}, bla_{OXY-2}, bla_{OXY-4}, bla_{OXY-5} and bla_{OXY-6} resistance genes, which lifted their resistance score to 1, although these genes are described as typically intrinsic among this SC. Few other AMR gene were detected, such as to streptomycin (*str*AB) in two *K. pasteurii*, with one of them also harboring the sulphonamide resistance gene *sul*2.

On the other hand, several virulence features and strains with high virulence score were found. Although no virulence loci were found in *K. planticola* isolates (score 0), yersiniabactin was detected in the 68% (n=26) of KoSC isolates and in all *K. ornithinolytica* genomes (consistent with Thorpe et al., 2022), which were classified as virulence score 1. The highest scores were assigned to *K. pneumoniae* sensu stricto isolates, with 9 isolates of virulence score 3 carrying the acquired *iuc3* (aerobactin sequence type AbST43) and 7 isolates of virulence score 4 harboring *iuc3* (with a novel combination of *iuc* alleles defined as AbST96) in combination with the yersiniabactin *ybt16* locus. The yersiniabactin profile presented a novel allele variant which was curated in BIGSdb-Pasteur and defined as YbST600. Virulence score 3 comprised all isolates of the ST3254 clone and the ST305 isolate, while all isolates of the ST4242 clone and the ST107 isolate showed virulence score 4.

Notably, *ybt16* was located on the integrative conjugative element ICEKp12, while *iuc3* was observed on a conjugative plasmid sequence with IncFIB/IncFII replicon type, classified as pMLST FIIK_2 in ST4242 isolates and FIIK_10 in ST3254 and ST107 isolates (Supplementary Table S9). Given the critical role of aerobactin in virulence and invasive disease, we compared the $iuc3^+$ plasmid sequences of our *K. pneumoniae* isolates with three $iuc3^+$ plasmid types found in *K. pneumoniae* isolated from pig livestock and hospitalized patients in the nearby area of Pavia city (Northern Italy) between 2018 and 2017 (Thorpe et al., 2022). We found a very high similarity (ANI>98%) among all sequences (Supplementary Table S10) and similar pMLST profiles. Gene cluster comparison (https://github.com/gamcil/clinker) of one annotated sequence for each replicon type/pMLST combination (Supplementary Table S11) and a close circular plasmid p90CM2-172k (ANI 97%; ~70x coverage) from a public strain (accession number NZ_CP071821) showed a high conservation of the *iuc*3 locus (*iucABCD*/*iutA*) in strains from our food facilities with those from a clinical case (699_C1) and a pig (2530_C2 and 1871_C1) collected in the same geographical area (Figure 3).

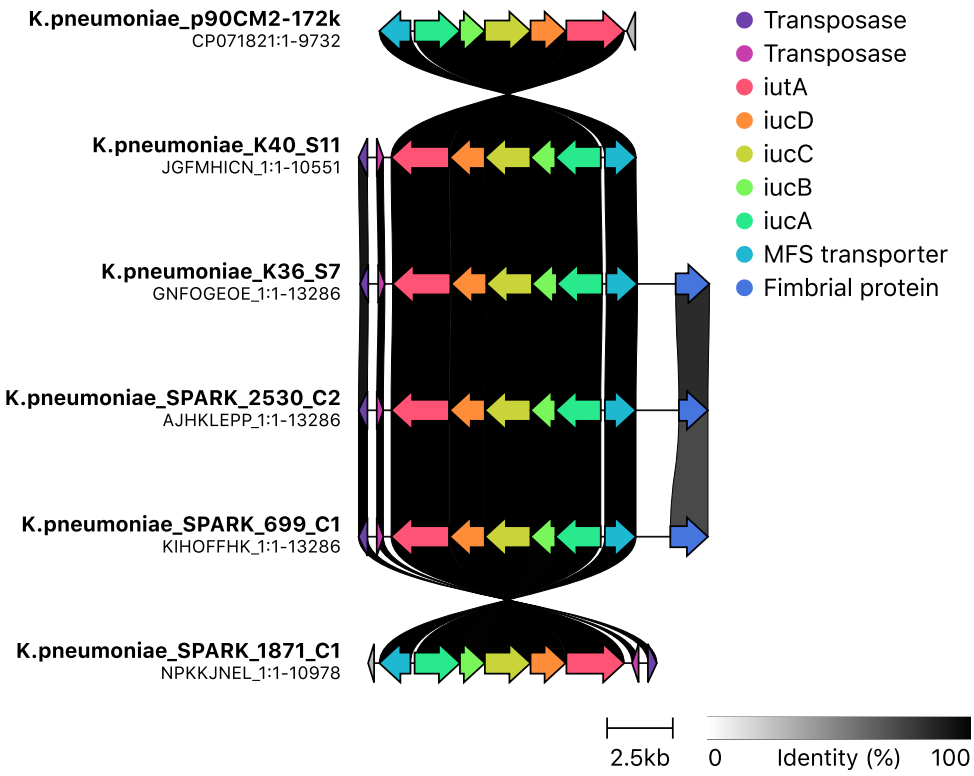


Figure 3: Clinker visualization showing gene cluster comparison of the plasmid-encoded aerobactin locus *iuc*3 (*iucABCD, iutA*) and other flanking genes encoding for transposable elements (transposase), membrane transporters (Major Facilitator Superfamily (MFS) transporters) as well as fimbrial proteins. Gene clusters belonged to close circular plasmid reference (K.pneumoniae_p90CM2-172k) from a public strain, salami (K.pneumoniae_K40_S11; K.pneumoniae_K36_S7), pig farming (K.pneumoniae_SPARK_2530_C2; K.pneumoniae_SPARK_1871_C1) and clinical settings (K.pneumoniae_SPARK_699_C1).

Beside a heterogenous gene repertoire, several strains harbor same gene clusters

Pan-genome analysis performed on the 73 *Klebsiella* spp. genomes clustered all protein-coding sequences into 19,365 groups of orthologues (Supplementary Fig. S2). Of these, only 15% (n=2,911) were conserved across isolates from different species. By contrast, 85% (n=16,454) of genes represented the accessory genome and were distributed among shell (n=5,802) and cloud (n=10,652) genes, respectively found in 15-95% and less than 15% of genomes. The cloud genes accounted for the 55% of all genes detected across the full set of genomes. Despite the heterogeneity of the gene repertoire, we observed several clusters of isolates harboring the same groups of orthologues (Supplementary Fig. S2).

Discussion and conclusions

AMR is a global public health threat strongly heightened by the dissemination of clinically relevant antimicrobial resistance genes and pathogens between clinical, community, agricultural and environmental settings (Larsson & Flach, 2022). The food-producing environment represent a possible reservoir of antimicrobial-resistant bacteria which can spread throughout the food chain, posing a potential hazard to public health (BIOHAZ, 2021). The ability of *Klebsiella* species to act

as a key trafficker of AMR genes following its wide ecological spread, the high AMR gene diversity and plasmid load (Wyres & Holt, 2018) combined with the increasing reports suggests a foodborne transmission capacity (Guo et al., 2016; Hartantyo et al., 2020; Hu et al., 2021; Theocharidi et al., 2022), emphasizing the importance of monitoring the spread of such species through the food chain. In the present study, we investigated the microbiological hazards of Italian RTE food artisanal production systems, an unexplored environment so far, to study their potential role as a source of *Klebsiella* spp. strains with pathogenic potential. We focused our investigation on cow milk-based soft cheese and pork meat-based salami because these are among the most consumed RTE food subcategories in the EU and are often associated with foodborne diseases (European Food Safety Authority & European Centre for Disease Prevention and Control, 2022).

Across the 1,170 samples collected, we observed a low prevalence of *Klebsiella* spp. (6%), with 73 strains identified among the two facilities considered together. Identification results from conventional and genomic methods agreed for only 72% of strains, confirming that biochemical and multiplex-PCR tests, commonly applied in food laboratories, are not as reliable as WGS, especially to distinguish close relatives within *Klebsiella* species complexes. While *K. ornithinolytica* and *K. planticola* strains were rare and associated with a specific production (cheese and salami respectively), KoSC and KpSC strains occurred in both production systems. However, 94% of KpSC strains were observed in the salami production, whereas 71% of KoSC were found in cheese production. *K. oxytoca*, *K. pneumoniae*, *K. michiganensis* or *K. ornithinolytica* strains have been already found worldwide in dairy and meat products (György & Laslo, 2021; Jin et al., 2018; Mladenović et al., 2018; Ogbolu et al., 2014; Uraz et al., 2008), and their associated raw materials and production environments (Gelbíčová et al., 2021). *Klebsiella* spp. were also isolated from minced pork meat fermentation processes in Belgium (Charmpi et al., 2020) and in Spanish raw pork sausages before the ripening process (Roig-Sagues et al., 1996). Our observations corroborate such findings and suggest that *Klebsiella* spp. clones can colonize dairy and meat food productions over several months. Such strains may come from the animal milking, slaughtering, and/or other processes at farm level, or persist in the facilities through contaminated environment or operators. Here, we provide the first evidence on the presence of several *Klebsiella* spp. populations in two Italian artisanal productions of ready-to-eat foods. Despite the high heterogeneity and genetic diversity at the strain level, which is typical for such populations (Holt et al., 2015; Wyres & Holt, 2018), we found that several clones were contaminating both processing environments and food products. Some of the detected clonal genotypes (e.g., new6 of *K. ornithinolytica*) lasted for several weeks or months along the whole production (from raw material to the final product), showing the capacity of *Klebsiella* to persist in this environment. Whether strains were repeatedly introduced in the artisanal food processing from the livestock or transmitted along the production by the food operators or persisting in the facilities due to cleaning and disinfection failures, will need to be addressed in future studies.

Although data from the food sector are still limited, events of genes' acquisition/loss in *Klebsiella* spp. population through horizontal gene transfer (HGT) are frequent during niche adaptation (Wyres et al., 2020). In a clinical context, the presence of mobile genetic elements (MGE) carrying virulence and antibiotic and metal resistance genes confers an advantage during host colonization or infection

(Rocha et al., 2022). Indeed, chromosomal recombination processes and acquisition of various AMR genes on diverse plasmids can shape the evolution of MDR clonal groups, such as CG258 (Wyres et al., 2019). The large repertoire of accessory genes (encompassing several encoding for virulence functions linked to invasive disease), that characterizes animal and human KpSC strains collected worldwide, emphasizes the considerable genomic plasticity of such species (Holt et al., 2015). This also emphasizes the need of gathering additional data on the distribution of concerning genes in strains from other important sources, such as food, to better understand horizontal gene transfer dynamics and *Klebsiella* spp. niche adaptation.

Our study showed that the virulence locus *iuc*3 was located on a large conjugative plasmid that has been likely transferred within and between genotypes contaminating the Italian facilities.

Investigating the genomic context of *iuc3* raised the possibility of a hypothetical transmission of virulence traits between isolates collected from different settings (food, clinical, environmental), while providing insights on the potential public health risk of *Klebsiella* isolated from artisanal food. The fact that *iuc3*-harbouring plasmids from the artisanal food chain were similar (>97%) to plasmid sequences of *K. pneumoniae* strains isolated from human and pig livestock in close geographical areas, may suggest a possible transfer of virulence plasmids across such sectors. A recent genome-based analysis performed on animals and food products in Germany (Klaper et al., 2021) proposed domestic pigs as a reservoir for *K. pneumoniae* plasmids carrying *iuc*3. Our results support this hypothesis, providing evidence on the circulation of *iuc*3^{+} *K. pneumoniae* clones in the raw pork meat and its processing environment, and report such strains also in the soft cheese (final product). We have no evidence of possible contact points between the two Italian food producers that could explain the presence of such plasmid-carrying genotypes in both facilities. However, given the restricted geographical area in which the facilities are located, the variety of sources (e.g., effluents and other residues as supposed by BIOHAZ, 2021) by which pathogens may enter the food-producing environments, and the ubiquitous nature of *Klebsiella* species (Thorpe et al., 2022), we could imagine that the environment may have played a role as a vehicle for spread of *K. pneumoniae* ST3254 across both food settings.

Analyzing local data within a broader ecological context is essential to understand the global circulation of genotypes across different niches or hosts. The cgMLST typing approach allowed us to compare local strains in the context of hundreds of public *Klebsiella* spp. sequences from other sources. We observed that high core genome proximity may exist between human and food strains of *K. pneumoniae* or *K. oxytoca* (~1.5% of variant differences). This suggests that in addition to the human-animal transmission of *Kp* strains (Dereeper et al., 2022; Thorpe et al., 2022), transmission may also occur from food to humans; however, more sampling and prospective designs will be needed to define the directionality of transmission.

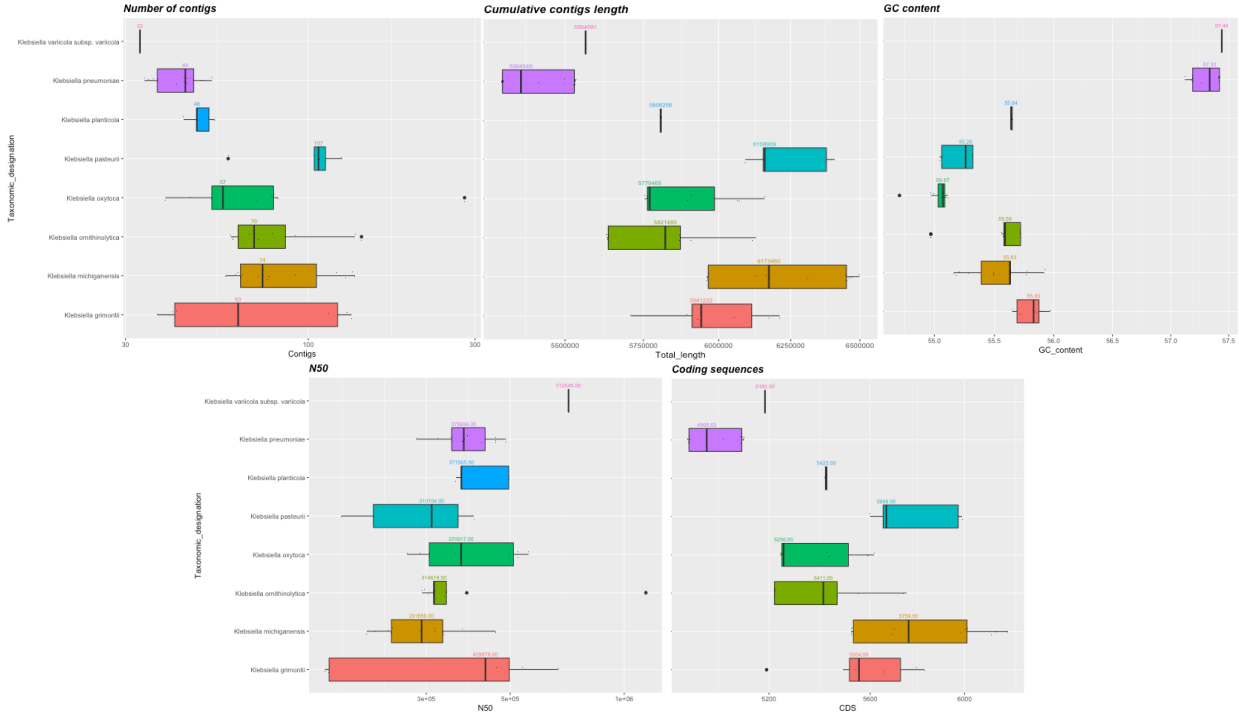
In conclusion, this study contributed to extend the body of knowledge on the prevalence and features of concern of *Klebsiella* spp. strains in the food chain. Following a one health approach, we provide evidence of *Klebsiella* presence and circulation of closely related genotypes and plasmid-encoded genes between interconnected settings (farm, food, and human). The carriage of AMR and virulence traits by *Klebsiella* along the food chain should not be underestimated, as such features could be

transferred to other bacteria in the same niche (e.g., human gut or environment) and help them colonize the gut of individuals who might develop invasive diseases. More active surveillance of *Klebsiella* in the food chain would help to gain more knowledge on *Klebsiella*, especially *K. pneumoniae*, as a foodborne pathogen.

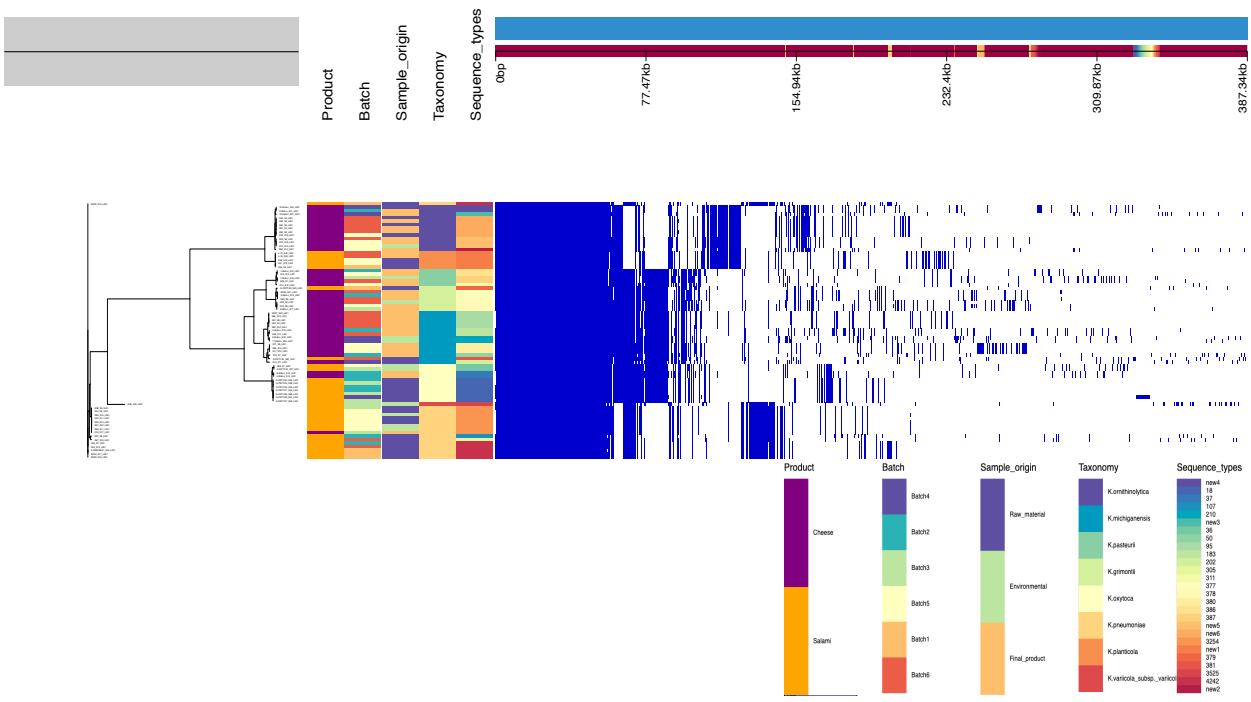
Data Availability

The paired-end reads included in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB56668 (https://www.ebi.ac.uk/ena/browser/view/PRJEB56668). The KoSC and KplaSC schemes were deposited in a Zenodo repository for public access (10.5281/zenodo.7477602). Supplementary materials are accessible in a dedicated GitHub repository under the following link: https://github.com/ceciliacrippa/Thesis_Supplementary_tables_Study3. Spreadsheets have been uploaded under file names "Supplementary_Tables_study3".

Supplementary material



Supplementary Figure S1: Boxplots of assembly (number of contigs, cumulative contigs length, GC content and N50) and annotation (coding sequences) quality metrics for *Klebsiella* spp. strains belonging to each species complex. The boxes display the median (50%) as well as the first (25%, Q1) and the third (75%, Q3) quantile. Values that are either less than Q1 – 1,5 * IQR or greater than Q3 + 1,5 * IQR are considered outliers.



Supplementary Figure S2: Interactive pangenome visualization of 73 *Klebsiella* spp. strains obtained by Phandango website. A core-genes phylogenetic tree is inferred with strains associated metadata (food product, batch, sample origin, taxonomy and STs) as well as pangenome content (core and accessory genes).

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Title: Genomic features of *Klebsiella* isolates from artisanal ready-to-eat food production facilities

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Discussion and conclusions

Thus far, artisanal fermented food productions have been key elements of the worldwide culture, identity, and heritage, and gained high popularity driven by richness of taste, high nutritional value and ethical commitments. However, the absence of full standardized production processes combined with inadequate monitoring of environmental and processing parameters across small scale plants, expose the artisanal food chain to several hazards for human health. Biological hazards such as *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus, Escherichia coli* O157:H7 and other *Enterobacteriaceae* may indeed disseminate along the whole food production continuum during manufacturing, processing and storage until the food retail, following direct or cross-contamination events.

For the purpose of this PhD thesis, fermented dairy and meat artisanal food productions, typically consumed in the Mediterranean countries, have been investigated by collecting samples from several food matrices (raw materials, semi-finished and final products) and environmental sites along their whole production chains between 2019 and 2021.

In the first study, the microbial safety of Italian artisanal soft cheese and salami processed in summer and winter seasons has been monitored by investigating the load of hygiene microbial indicators and main biological hazards. In cheese, seasonality's variation significantly affected bacterial growth, highlighting in winter season an increase of TBC and LAB at the end of the cheese storage, when exposed to abuse temperatures. Since raw materials (milk) were not contaminated, it appears that the lowered microbiological quality observed during winter likely fostered the dissemination of *S. aureus* and *K. oxytoca* strains to cheese via cross-contamination from contact surfaces or hands of operators. By contrast, in salami the fermentation processes, driven by the natural evolution of indigenous starter cultures over 6 months of ripening, has leaded to LAB and TBC increase which showed higher load in summer, alongside a reduction of *Enterobacteriaceae* and water activity. Although *S. aureus* and *K. pneumoniae* strains contaminated raw materials, semi-finished salami and environmental sites, the absence of microbiological hazards in final products suggested the 6-month ripening as an effective control measure. Neither cheese nor salami represented vehicles of foodborne pathogens such as *L. monocytogenes*, *Salmonella* or VTEC. This suggests that combining thermal milk treatment with the use of bio-protective cultures in cheese as well as the autochthonous microbial populations and technological parameters in salami hurdles microbiological hazards while preserving quality of these artisanal products. Nevertheless, the spread of *S. aureus* and *Klebsiella* spp. on ready-to-eat products such as salami and cheese raises concerns regarding consumer's safety, although further studies are needed to fully explore their pathogenic traits. It is anyway alarming that bacterial species of clinical concern such as *K. pneumoniae* may be present in the food chain but often go unnoticed as not considered a foodborne pathogen.

The second study focused on genomic investigations of a broader range of batches and artisanal fermented meat and dairy productions typically consumed in Mediterranean countries (Portugal, Spain, Italy and Morocco) confirming a total of 42 foodborne pathogen strains belonging to *L. monocytogenes*, *S. enterica* and *S. aureus*. Combining base-by-base (SNPs calling) and gene-by-gene

(*in silico* 7-loci MLST) approaches, the circulation of different clonal groups of strains was observed in the same artisanal production plant over several months, confirming the ability of these pathogens to contaminate, spread and persist in the artisanal productions. Of particular concern were (i) the dissemination of *L. monocytogenes* ST1 and ST8 clones carrying several pathogenic features through the environment and fermented Spanish sausage, (ii) one *S.* Paratyphi B ST43 clone persisting in the Portuguese meat processing environment and final products over one year exhibiting a high repertoire of virulence genes and (iii) the occurrence of several cross-contamination events that introduced genotypically diverse *S. aureus* in artisanal meat and cheese facilities of Spain, Italy and Morocco over time, with ST121 carrying the higher number of virulent traits. In addition, the presence of several genes linked to AMR resistance identified on *S. enterica* serovar Hadar and Senftenberg isolates from Moroccan fermented sausage emphasizes the potential contribution of such strains to the spread of AMR through the food chain. The multi-countries investigations of different local artisanal productions of animal origin provided further insights on the circulating bacterial foodborne pathogens genotypes and their pathogenic potential, thereby confirming that these artisanal foods may expose the consumer to microbial hazards (*L. monocytogenes*, *S. enterica* and *S. aureus*). A natural progression of this work could be to speculate possible transmission dynamics of pathogenic and AMR features between foodborne strains and other microbial populations or ecological niches.

The microbiological insights reported in the first study provided an interesting dataset to decipher the spread and long-term detection of *Klebsiella* spp. strains along the Italian soft cheese and salami artisanal facilities by using advanced genomic methods. Few studies focused on *K. pneumoniae* strains with antimicrobial resistance and virulence traits from food and farm animals, raising concerns about the potential role of *Klebsiella* spp. as a foodborne pathogen, which may also act as a key vehicle of virulence and AMR spread. However, very little was found in the literature on prevalence rates of antibiotic resistant and/or virulent *Klebsiella* spp. from artisanal food chains, suggesting the dairy and meat artisanal productions is a hitherto unexplored food niche. Here, the potential role of Italian soft cheese and salami food productions as a source of *Klebsiella* spp. strains with pathogenic potential has been then investigated on a large collection of samples across six batches.

Results from this study has confirmed that WGS is an excellent and more reliable technology to assess strain's taxonomy and distinguish close relatives within *Klebsiella* species complexes compared to conventional biochemical and multiplex-PCR tests commonly applied in food laboratories. Although a low prevalence of *Klebsiella* spp. has been observed across the two facilities, several clones were found contaminating both processing environments and food products for weeks or months. These observations corroborated previous studies on *Klebsiella* spp. populations worldwide found from dairy and meat products and provided additional evidence on their presence in Italian artisanal productions of ready-to-eat foods. Future research will need to clarify whether strains were repeatedly introduced in the artisanal food processing from the livestock or transmitted along the production by the food operators or persisting in the facilities due to cleaning and disinfection failures. However, exploring the genomic context of the virulence key locus *iuc3*, a hypothetical transmission of virulence traits between *K. pneumoniae* isolates from different settings (food, livestock, clinical,

environmental) was emphasized, providing new insights on the circulation of similar virulence plasmids in the artisanal facilities, a geographically close pig livestock and a human clinical case.
Finally, by means of cgMLST analysis it has been possible to compare local strains within a broader ecological context of hundreds of public *Klebsiella* spp. sequences from other sources following a One Health approach. High core genome proximity has been found between human and food strains of *K. pneumoniae* or *K. oxytoca* (~1.5% of variant differences), suggesting that in addition to human-animal transmissions, food-human transmission of *Kp* strains may also occur, whilst more sampling and prospective designs are needed to define the directionality of transmission.
In conclusion, the evidence of *Klebsiella* presence and circulation of closely related genotypes and similar virulence plasmids between interconnected settings (food producing animals, food, and human) reported in this study underlines that more active surveillance in the food chain is needed to converge a deeper knowledge on *Klebsiella*, especially *K. pneumoniae*, as a potential foodborne pathogen.
Overall, a major finding from this PhD thesis emphasizes that the challenging standardization of processing and environmental surroundings in artisanal productions of animal origins might increase the risk associated to cross-contaminations within the facility. Contamination's exposure may occur during harvest or slaughtering, processing, storage, and packaging, suggesting that the production environment might be an important contributor through animal feces, soil, air, feed, water, equipment, animal hides and personnel. This work also confirms that WGS provides an excellent one-stop solution to apply advanced genome approaches to investigate the diversity, dynamics and spread of bacterial pathogens strains and virulence and AMR traits along the artisanal food chain.
Global findings from this thesis finally suggest that enhancing surveillance programmes and adopting cutting-edge technique like WGS will help to reduce the food safety risks associated with the dairy and meat production chains and better inform stakeholder and policy-makers. A continuous system of preventive measures would be required, from the monitoring and control of animal feed and progressing, through good farming practices and on-farm controls, good manufacturing and hygiene practices, consumer safety awareness, to proper implementation of food safety management throughout the entire food chain. Food safety authorities and national governments must support the definition, application and control of such systems from a technical and financial perspective.

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