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XXXV CICLO

Environmental Microbiome Mapping in the Food Industry: a Strategy to Improve Food Quality and Safety

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CHAPTER 1

Overview

1.1. Thesis presentation

To ensure quality and safety of food products, food business owners adopt routinary cleaning and disinfection procedures in their food manufactures. The purpose of such procedures is to remove food residuals and reduce the adherence of microorganisms on surfaces. However, even after these procedures, a low amount of bacteria resist on tools, equipment and machines. Some of these microbes occurring after the cleaning and disinfection are transient, whereas others become resident and specific to the facility. One strategy that bacteria use to establish on surfaces is through the production of biofilms, which further protect microbes from disinfectants and detergents and enhance the transmission of antimicrobial resistance genes, thus contributing to the current burden of antibiotic resistance worldwide.

Currently, international regulations do not pose limits in terms of residual contaminations after the cleaning and disinfection procedures (with some exceptions), and food business operators verify the efficiency of cleaning/disinfection as part of the implementation of HACCP-based procedures. To date, methodologies used by food business operators to verify the efficiency of such procedures require the isolation of microorganisms and on the phenotypic characterization of isolates, as well as on the total viable count.

However, these methods suffer from several limitations. Cultural-dependent methods, i.e., those procedures that require the use of culture media, are quite slow, since more than a week might be required for the identification of some isolates and the detection of microbial activities. Also, false-negative results might occur, e.g., because of viable but non-culturable (VBNC) microorganisms that are metabolically active, despite their inability to grow on plate. Finally, culture-dependent

methods are unable to target those species defined as ‘unculturable’, for which culture media have not been developed yet or that show very slow growth rates.

Therefore, novel procedures that aim at ensuring the efficiency of cleaning and disinfection and to describe the communities residing in food industries with a high resolution are strongly needed. Such procedures might help in detecting pitfalls in the sanitation of the production area, thus helping food business operators to adopt focused choices and to prevent food waste, making the food manufacturing more sustainable.

In this regard, metagenomics, i.e., methods based on the high throughput sequencing (HTS) of nucleic acids extracted from whole microbial communities, revolutionized the way to study microorganisms from very diverse environments. Indeed, these technologies unraveled the existence of previously uncharacterized taxa, also providing new information about their metabolic potential. In addition, thanks to incessant technologic improvements, time of analysis are drastically lower compared to culture-dependent techniques.

Therefore, this thesis aims to validate a procedure based on the HTS-based mapping of microbial communities residing in the food industry in order to assess the composition of the communities (at species- and strain-level) and their potential outcomes for food quality and safety. Such procedure might represent a new tool for food business operators, which might better assess the efficacy of their cleaning and disinfection procedures.

We applied the procedure in several food industry settings. Indeed, **Chapter 3** and **Chapter 4** discuss the results obtained after the application of metagenomic sequencing strategies in facilities producing minimally processed vegetables and ice creams respectively, with a major focus on the presence of pathogens in the food manufacturing and also on the presence of virulence and antibiotic resistance genes.

Chapter 5 focuses on microorganisms residing on surfaces in cheesemaking facilities from 4 European countries producing several kinds of cheeses. In the chapter, the potential beneficial

outcomes of these microbes are discussed, with a focus on bacteriocins production and on facility-specific strain-level diversity.

Furthermore, **Chapter 6** reports the results of a metagenomic-based large-scale analysis of the food processing environment from several industry types, particularly focusing on antibiotic resistance and transmission potential.

Finally, the **Conclusion** chapter focuses on future perspectives and on the feasibility of the application of such procedure as a routinary monitoring of contamination of surfaces after cleaning and disinfection.

1.2. Presentazione della tesi

Gli operatori del settore alimentare effettuano detersione e disinfezione di routine nelle loro aziende come parte delle strategie atte a garantire qualità e sicurezza degli alimenti. L'obiettivo di queste procedure è rimuovere i residui di alimenti e limitare l'aderenza di microbi sulle superfici, sugli strumenti e sui macchinari. Tuttavia, è noto che basse concentrazioni di microrganismi persistono sulle superfici a contatto con gli alimenti anche dopo l'applicazione delle procedure di sanificazione. Alcuni di questi microbi sono transienti, mentre altri diventano residenti, adattandosi specificamente alle condizioni presenti nello stabilimento. Una delle strategie che i microrganismi adottano per diventare residenti è la produzione di biofilm, il quale offre loro protezione contro disinfettanti e detergenti, favorendo invece la trasmissione di geni di resistenza ad antibiotici, contribuendo ad aggravare la crisi mondiale.

Ad oggi non vi sono limiti di legge in termini di contaminazione microbica residuale successiva alle procedure di pulizia e disinfezione (anche se con alcune eccezioni), e gli operatori del settore alimentare verificano l'efficacia di dette procedure attraverso piani di monitoraggio basati sui principi dell'HACCP. Al momento, i metodi usati dagli operatori del settore alimentare per verificare l'efficacia di detersione e disinfezione si basano sull'isolamento dei microrganismi e

sulla loro caratterizzazione fenotipica, nonché sul conteggio della carica microbica totale. Tuttavia, questi metodi hanno alcuni limiti.

I metodi coltura-dipendenti, cioè le procedure che richiedono l'utilizzo di mezzi di coltura, sono lenti, dal momento che per l'identificazione e la caratterizzazione metabolica di un isolato può essere necessaria più di una settimana. Inoltre, con le metodologie coltura-dipendenti si possono riscontrare falsi negativi, poiché i microrganismi vitali ma non coltivabili sono metabolicamente attivi, pur non essendo in grado di crescere in piastra. Infine, detti metodi sono incapaci di isolare le specie definite come 'non coltivabili', per le quali non sono ancora stati messi a punto terreni di coltura ad-hoc o che crescono molto lentamente.

Pertanto, è indispensabile sviluppare nuove procedure finalizzate a verificare l'efficacia di detersione e disinfezione e a descrivere nel dettaglio le comunità che risiedono nelle industrie alimentari. Tali procedure potrebbero evidenziare punti deboli nelle procedure di sanificazione, così da aiutare gli operatori del settore alimentare a effettuare scelte mirate e a prevenire gli sprechi alimentari, rendendo l'industria alimentare più sostenibile.

A tal proposito, la metagenomica, cioè il sequenziamento ad alto rendimento degli acidi nucleici estratti da intere comunità microbiche, rappresenta una rivoluzione. Infatti, questo approccio ha permesso la scoperta di microrganismi precedentemente mai caratterizzati, fornendo anche nuove informazioni sul loro potenziale metabolico. Inoltre, grazie al continuo progresso tecnologico, i tempi di queste analisi sono notevolmente inferiori rispetto a quelli richiesti dalle tecniche coltura-dipendenti.

Pertanto, l'obiettivo di questa tesi è quello di validare una procedura basata sul sequenziamento del DNA microbico raccolto nelle industrie alimentare con l'obiettivo di analizzare la struttura delle comunità (a livello di specie e di ceppo) e le loro potenziali implicazioni sulla qualità e sicurezza degli alimenti. Questa procedura potrebbe rappresentare un nuovo strumento per gli operatori del settore alimentare per verificare con maggiore rapidità e accuratezza l'efficacia delle loro procedure di detersione e disinfezione.

La procedura è stata applicata in diversi tipi di industria alimentare. I **Capitoli 3 e 4** discutono rispettivamente i risultati ottenuti dopo l'applicazione delle tecnologie di sequenziamento ad alto rendimento in aziende che producono vegetali minimamente processati e gelati, focalizzando l'attenzione sulla presenza di patogeni e di geni associati a virulenza e antibiotico-resistenza.

Il **Capitolo 5** si concentra sui microrganismi stabilizzatisi in caseifici appartenenti a 4 Stati europei. In questo capitolo sono stati discussi i potenziali benefici di questi microbi, con un focus sulla produzione di batteriocine e sulla diversità a livello di ceppo specifica di ciascun'azienda.

Inoltre, il **Capitolo 6** riporta i risultati ottenuti dall'analisi metagenomica su larga scala dell'ambiente di produzione di diversi tipi di industrie alimentari, concentrandosi sull'antibiotico-resistenza e sulla sua potenziale trasmissibilità.

Infine, il capitolo delle **Conclusioni** discute le prospettive future e la fattibilità dell'applicazione di queste procedure per il monitoraggio della contaminazione delle superfici dopo la detersione e la disinfezione.

CHAPTER 2

Literature review

2.1. Food processing facilities are inhabited by a resident microbiome

Microbial contamination in food processing environments considerably influences food quality and safety. In food industries, an environmentally-adapted microbiome can colonize the surfaces of equipment and tools and be transferred to the food product or intermediates of production during handling, manufacture, processing and storage. Indeed, food contact surfaces often represent a good niche for microorganisms to persist and, indeed, proliferate. Moreover, non-food contact surfaces are also potential reservoirs of microbes, which over a longer term can be sources of food contamination. Although frequent cleaning and disinfection procedures are routinely implemented in all food industries, it is recognized that these are not always effective in eliminating the resident microbial consortia specific to each food plant (Griffith, 2005). Such microbial populations are well-adapted to the specific environmental conditions that they are exposed to and tend to develop, often as biofilms, on surfaces that are particularly difficult to clean due to challenges relating to access, surface irregularities or the retention of sticky materials. These microbes can then proliferate due to the availability of food residues and exudates in such micro-environments and can ultimately represent a possible source of pathogens or spoilage-associated microbes that can lead to cross-contamination of foods.

According to recent reports, one third of all the food produced worldwide is wasted every year, accounting for ~1.3 billion tons. Industrial food processing is among the factors contributing the most, producing 19% of the yearly food wastes (de los Mozoz et al., 2020). Food wastes produced by the industry include not only processing wastes, but also ingredients/products spoiled by microorganisms. Although international organizations are making efforts to promote good practices and reduce food waste, there is still the need of validated SOPs specifically developed for the food industry to improve efficiency of production and limit spoilage-related food loss, in

order to make food production more sustainable. In this regard, metagenomics might help food business operators to reduce the occurrence and the establishment of potentially spoilage microbes. In the past years, metagenomics has begun popular for microbiome mapping in food handling or processing facilities (Table 2.1). This approach has been primarily applied in dairies and, to a lesser extent, raw meat processing environments (e.g., butchers, facilities producing fresh sausages). All of these studies clearly showed that food processing environments are inhabited by a resident microbiome that persist despite routine cleaning practices and may be easily transferred to the final food product. Indeed, the studies to date suggest that most of the taxa found in processing environments are also found in food products produced in that facility (Table 2.1).

The environmental microbiome may represent a primary source of contamination in facilities where fresh products are produced or handled, such as raw meat and fish (Hultman et al., 2015; Stellato et al., 2016; De Filippis et al., 2013; Møretrø et al., 2016), ready-to-eat, composite meals (Pothakos et al., 2015) and fresh fruit (Tan et al., 2019). For instance, meat processing environments are often contaminated by well-known microbial spoilers (*Brochothrix thermosphacta*, *Pseudomonas* spp., lactic acid bacteria) that are transferred to the product and then selected for by the storage conditions, e.g., temperature, gaseous atmosphere employed. Moreover, some studies also report the presence of potential pathogens (e.g., *Salmonella*, *Escherichia coli*, *Listeria monocytogenes, Staphylococcus* spp.) or undesirable gene families (e.g., antimicrobial resistance genes) on food processing surfaces, which may contaminate the food product. These hazardous microbes may then proliferate when they find the appropriate conditions (Table 2.1). Nevertheless, the environmental microbiome may also be a reservoir of beneficial microbes that contribute to the food manufacture process, especially in the case of fermented foods (Table 2.1). This was highlighted in several studies involving fermented dairy products or beverages (Table 2.1). Dairies usually harbor lactic acid bacteria and other microbes important for ripening of specific cheeses (e.g., *Debaryomyces, Brevibacterium, Corynebacterium*; relevant to smear-ripened cheese maturation), while the environments of wineries and breweries can be a source of

Saccharomyces cerevisiae and other yeasts involved in fermentation to produce alcoholic beverages (Table 2.1). Also, microorganisms residing on food contact surfaces may exert an antimicrobial activity against pathogens such as *Staphylococcus aureus* and *L. monocytogenes*, by competing for nutrients and producing bacteriocins or other antimicrobial compounds (Son et al., 2016; Castellano et al., 2017). However, it should be pointed out that most of the studies available focused on just 1 or 2 different facilities. Thus, a wide-scale and systematic analysis of food environmental microbiomes would be necessary to encourage the implementation of microbiome mapping procedures in food industries as an additional tool to support overall quality and safety management systems.

Table 2.1 Studies using HTS to map microbial communities in food manufacturing facilities.

Type of food industry	**Number of facilities sampled**	**Dominant taxa (environment)**	**Dominant taxa were found in food?**	**Surfaces sampled**	**Detection of potential pathogens in the environment**	**Detection of beneficial microbes**	**Reference**
African fermented milk	120	*Lactobacillus, Streptococcus*	Yes	Wooden bowls	No	Yes	Parker et al, 2018
Bakery	4	*Saccharomyces cerevisiae, Weissella, Lactobacillus, Leuconostoc, Bacillus, Streptococcus, Pseudomonas, Staphylococcus*	Yes	Dough mixer, storage boxes, walls	*Staphylococcus*	Yes	Minervini et al., 2015
Brewery	1	*Saccharomyces cerevisiae, Kocuria, Micrococcus, Acinetobacter, Pediococcus*	Yes	Fermentation tanks, drain, sink, barrels	No	Yes	Bokulich et al., 2015

Cheeses	1	*Leuconostoc citreum, Pseudomonas, Lactococcus lactis*	NA	Floor drains	*Listeria monocytogenes*	Yes	Dzieciol et al., 2016
Cheeses, pasta-filata	1	*Streptococcus thermophilus, Lactobacillus delbrueckii, Lactococcus lactis, Pseudomonas*	Yes	Curd vat, draining table, molding and stretching machines, knives, ripening room	No	Yes	Stellato et al., 2015
Cheeses, pasta-filata	1	*Macrococcus caseolyticus, Lactococcus lactis*	Yes	Curd vat, draining table, knives, brining tank, stretching and molding machines	No	Yes	Calasso et al., 2016
Cheeses	4	*Escherichia coli, Acinetobacter johnsonii, Salmonella enterica*	Yes	Curd vats, milk tanks, molds, floors, sink, drains	*E. coli, S. enterica*, antibiotic resistance genes	No	Alexa et al., 2020
Cheeses, smear-ripened	1	*Lactobacillus kefiranofaciens, Streptococcus thermophilus, Debaryomyces hansenii, Saccharomyces unisporus*	Yes	Floor drains	No	Yes	Schön et al., 2016
Cheeses, smear-ripened	2	*Debariomyces, Lactococcus, Staphylococcus, Brevibacterium*	Yes	Drains, aging racks, tanks, draining table	*Staphylococcus*	Yes	Bokulich et al., 2013

Cheeses, smear-ripened	1	*Streptococcus, Staphylococcus, Lactococcus, Pseudomonas*	Yes	Cow teats, milk tanks, molds, packaging, aging shelves	No	Yes	Falardeau et al., 2019
Cheeses, smear-ripened	1	*Brevibacterium, Corynebacterium, Debariomyces, Galactomyces*	Yes	Wooden aging shelves	No	Yes	Guzzon et al., 2017
Cheeses, washed rinds	2	*Halomonas, Corynebacterium, Staphylococcus, Brevibacterium*	Yes	Aging shelves and racks, walls, floors	*Staphylococcus*	Yes	Quijada et al., 2018
Chinese liquor	1	*Lactobacillus, Leuconostoc, Pseudomonas, Saccharomyces, Rhizopus, Rhizomucur*	Yes	Fermentation jar	*Staphylococcus*	Yes	Pang et al., 2018
Fruit packing	3	*Pseudomonadaceae, Flavobacteriaceae, Xanthomonadaceae, Aureobasidiaceae, Aspergillaceae*	Yes	Floors	*Listeria monocytogenes*	No	Tan et al., 2019
Milk	1	*Lactococcus, Acinetobacter, Streptococcus, Staphylococcus, Bacillus*	Yes	Silos, pasteurizers, concentrators	*Staphylococcus*	Yes	Kable et al., 2019
Milk		*Streptococcus, Pseudomonas, Staphylococcus, Enterobacteriaceae*	Yes	Tanker tucks	*Staphylococcus*	Yes	Kable et al., 2016
Raw meat, sausages	1	*Brochothrix, Leuconostoc, Lactobacillus, Yersinia*	Yes	Transport belt, meat emulsion blender, filling	*Yersinia*	No	Hultman et al., 2015

				machine, trolleys			
Raw meat, steaks	20	*Brochothrix, Pseudomonas, Psychrobacter, Streptococcus*	Yes	Chopping boards, knives, operator hands	No	No	Stellato et al., 2016
Raw meat, steaks	1	*Brochothrix, Pseudomonas, Psychrobacter, Streptococcus*	Yes	Chopping boards, knives, operator hands, cold-store walls, beef carcass	No	No	De Filippis et al., 2013
Ready-to-eat meals	2	*Leuconostoc, Lactobacillus, Streptococcaceae, Pseudomonas*	Yes	Mixing vessel, bench, carrier vessel, mixing machine, washing tank, dicer	No	No	Pothakos et al., 2016
Japanese rice liquor (sake)	1	*Saccharomyces cerevisiae, Aspergillus, Leuconostoc, Staphylococcu, Bacillus, Lactobacillaceae*	Yes	Fermentation tanks, aging tanks, mixing tub, drains, filter press, steamer	*Staphylococcus*	Yes	Bokulich et al., 2014
Salmon fillets	2	*Pseudomonas, Shewanella*	Yes	Seawater tanks, conveyors, gutting machine	No	No	Møretrø et al., 2016
Winery	1	*Saccharomyces cerevisiae, Hanseniaspora uvarum, Brevundimonas, Comamonadaceae, Enterobacteriaceae*	Yes	Grape crusher, press, fermentor, pump, barrels, drain	No	Yes	Bokulich et al., 2013

2.2. Metagenomics-based microbiome mapping in food processing environments

Microbial colonization of surfaces and tools in the food processing environments is a widespread phenomenon (Møretrø & Langsrud, 2017), but the structure or composition of the microbial communities may vary substantially in each food plant or in different sites of the same facility, influenced by the building layout (Figure 2.1). Moreover, several other factors may contribute to the number and composition of microbial populations on food contact surfaces, or influence the microbial dynamics thereof (Figure 2.1). Depending on their composition and hygienic conditions, ingredients, raw materials and processing water entering the food processing facility may introduce new microbial populations that might be different from lot-to-lot. Also, microbial sources along the food chain may include contaminated air (bioaerosols), an incorrect handling of industrial wastes and food industry operators (Figure 2.1). These populations might ultimately become resident in the environment when appropriate niches are found, but can also change over time in response to factors such as the presence of organic residues, variations in the cleaning and

Figure 2.1 Factors influencing environmental microbiome in food industry.

disinfection practices, temperature shifts (e.g., during different seasons) and other factors (Figure 2.1).

The development of high-throughput sequencing technologies (HTS) in recent years has provided the opportunity to explore microbial consortia at an unprecedented depth. These approaches can be successfully applied to environmental mapping activities in the food industry (Figure 2.2). When preparing for HTS-based profiling, amplicon- or shotgun-based approaches can be considered. For the former, a gene of taxonomic relevance, e.g., the 16S rRNA gene from bacteria, is amplified through PCR from total microbial DNA directly extracted from the sample. In this way, a description of the taxonomic composition of the microbiota in a given environment is obtained (Figure 2.2). There are some issues associated with this approach. Firstly, the presence of an amplification step may lead to a bias due to the preferential amplification of some taxa, distorting the quantitative and qualitative insights gained. This has been noted to be particularly troublesome for Fungi (De Filippis et al., 2017; De Filippis et al., 2018). In addition, different target genes must be sequenced to gain insights into different subpopulations of the microbiota (e.g., Bacteria, Fungi, Archaea, Protozoa), meaning that obtaining quantitative data across the respective populations is not possible. Many of these problems are overcome by using shotgun metagenomics (SM). In shotgun metagenomics (SM), total DNA is fragmented and sequenced without any prior selection steps. Therefore, fragmented microbial genomes of the entire microbial community are sequenced and a complete description of the microbial ecosystem is obtained, including of representatives from different categories of microorganisms, and also phage/viruses (Figure 2.2). In this case, in addition to the taxonomic composition of the microbial community, its genetic potential can be retrieved, providing the means to study the potential functions that a specific microbial community may harbour. In addition, microbial genomes of the most abundant strains can be reconstructed, allowing precious strain-level information to be gathered. Both approaches could be used by food companies to monitor the resident microbial populations in their facilities and to identify possible routes of contamination (Figure 2.2). The use of amplicon-based

HTS may be useful to evaluate the efficacy of cleaning practices, to track microbial contaminants (either spoilage or pathogenic microbes) on specific tools or equipment surfaces and evaluate how the processing plant microbiota changes over time or in response to the modification of processes (e.g., the introduction of novel cleaning practices, new suppliers or changes in the process parameters; Figure 2.1; McHugh et al., 2020). This approach, which is easier and cheaper but less informative than SM, may be introduced to support routine quality and safety management plans.

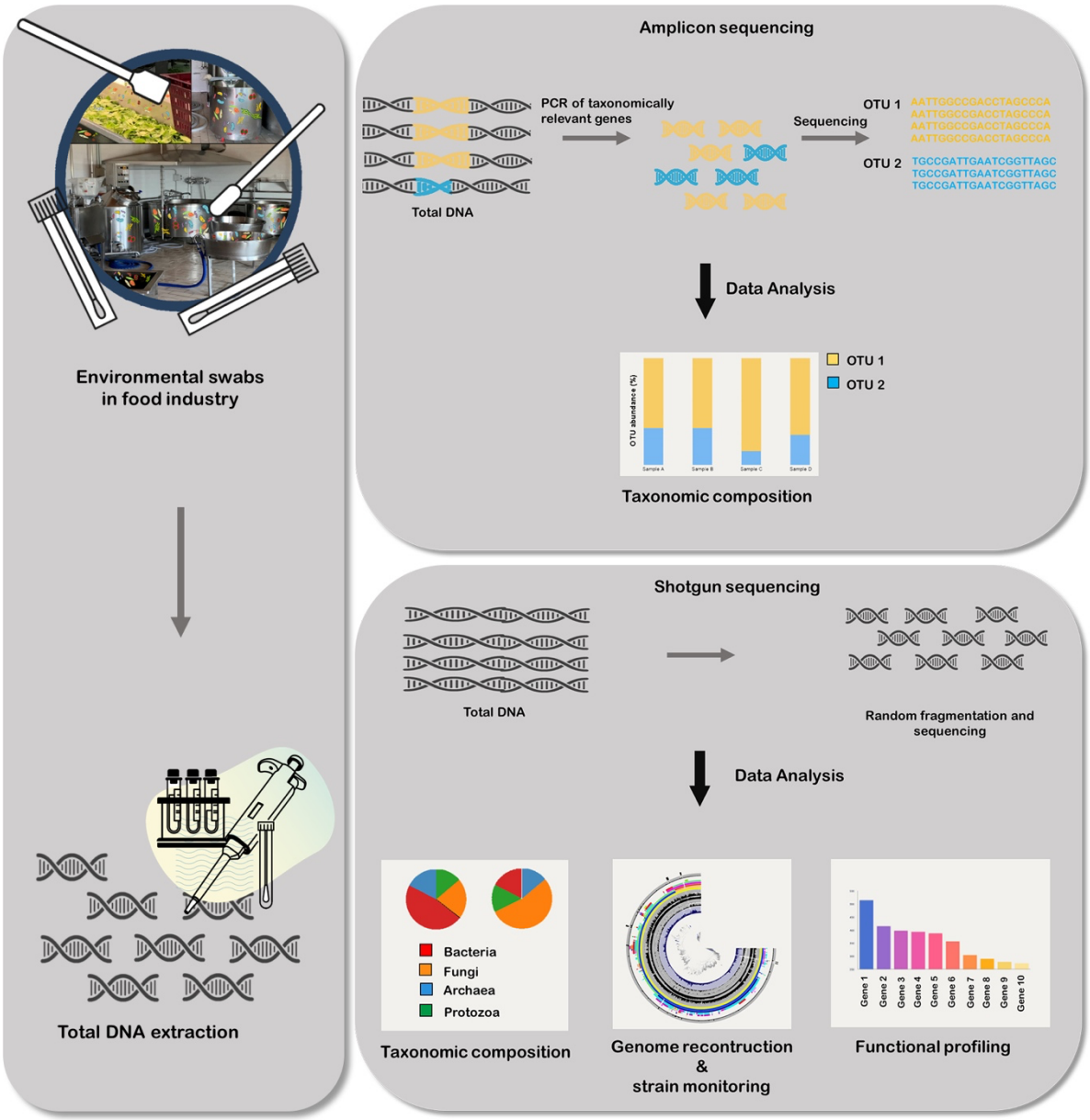

Figure 2.2 High-throughput sequencing approaches for microbiome mapping. Different high-throughput sequencing approaches for the study of environmental microbiome in food industry.

On the other hand, using SM, the company has the potential to go further to understand the functional potential of the microbial communities inhabiting its processing plant (to date mainly unexplored), identifying the presence of genes responsible for potentially dangerous activities and intervening in time to avoid the spread of undesirable microbes to the product. In this way, tracing of genes related to virulence or spoilage activities (e.g., antibiotic resistance, toxin production, biofilm production) in a food facility-associated microbiome is possible (Bokulich et al., 2015; Alexa et al., 2020). In addition, SM is better suited to detecting phage and identifying bacteria at the species level, the latter being particularly important when discriminating between pathogens (e.g., *L. monocytogenes*) and closely related non-pathogenic (e.g., *Listeria innocua*) species. Furthermore, since several microbial activities are strain-specific, strain-level monitoring may be achieved by SM to track starter-associated strains, as well as to identify spoilers or pathogens, and monitor strain persistence and/or evolution in the plant during time or in response to process changes (Figure 2.2). Strain-level tracking can be also extended to raw materials, intermediates of food processing and food products to identify at which stage during the process the contamination takes place and also to trace back the origin of the contaminating strains. Therefore, the application of SM for microbiome mapping in the food industry has the potential to revolutionize food safety and quality management systems.

In order to use these mapping approaches at industrial level, food industries should be first provided with appropriate standard operating procedures (SOPs) that, in combination, would represent an entire workflow. Considerable efforts have been made to standardize sampling procedures, sample storage and the subsequent steps in the analyses of microbiomes from other environments (e.g., human gut microbiome, http://www.microbiome-standards.org/). While such protocols are not yet available for food and food-related microbiomes, there are considerable merits in investing time to address this gap. SOPs can be developed de novo or adapted from existing protocols, followed by testing and validation in the food industry. The procedures will have to be versatile to reflect different processing environments and foods, including industries

involved in raw and processed meat and fish products, raw vegetables, fresh and ripened cheeses, fermented beverages and others. These foods will be susceptible to different possible types of microbial contamination as well as different routes of microbiota entry and establishment in the processing plant. Once validated SOPs are available, dissemination and demonstration activities will also be needed in order to lead to the widespread application of the developed SOPs and strategies by food business operators and laboratories undertaking outsourced environmental monitoring analyses. Public investment is needed to pursue these aims and specific innovative initiatives are currently ongoing in Europe to achieve this goal. One of such examples is MASTER (Microbiome applications for Sustainable Food Systems through Technologies and Enterprise; https://www.master-h2020.eu), an EU-funded collaborative innovation initiative aimed at implementing methodologies and SOPs from available microbiome data in order to provide the food industry with appropriate protocols that can be used to map the microbial contamination in the processing environments with the ultimate scope of process optimization, waste reduction and improvement of food quality and safety.

2.3. Limitations and technical warnings

There are a number of challenges that need to be overcome to harness the full potential of environmental microbiome mapping tools at food processing facilities. The major technical issue, especially for SM applications, is the recovery of an appropriate amount of DNA, of sufficient quality. Environmental mapping is usually carried out by swabbing industry equipment, tools and surfaces after routine cleaning. Therefore, the microbial loads on these surfaces may be very low, i.e, below 2.5 CFU/cm^2 in most cases (Griffith, 2005), thus limiting the amount of nucleic acids that can be obtained. For the higher amount of DNA required for SM, a prior whole-genome amplification may be used to increase the available DNA concentration. More specifically, a multiple displacement amplification (MDA) can be used, which is a non PCR-based technique that consists in the random amplification of the whole metagenome under isothermal conditions, using random exonuclease-resistant primers and the phi29 DNA polymerase (Yokouchi et al., 2006).

Although this provides a means of increasing workable DNA amounts, it is well-documented that this approach may represent a source of bias (Kim & Bae, 2011). Indeed, when comparing two popular MDA kits, Yilmaz et al. (2010) showed that both made quantitative comparisons unrealistic when compared with unamplified metagenomic samples.

Another point of primary importance with respect to optimising the recovery of microbial cells is the choice of the swab and the swabbing procedure (e.g., the width of the surface to be sampled). Several swab types are available on the market, which differ with respect to their shape and the material used (Table 2.2). Two main types of swab categories exist: swab tips or sponges. To improve the collection of microbial cells, the use of sponge swabs is recommended as these have a wider sampling surface. Cellulose-derived and synthetic are the most commonly used materials. Cellulose-derived swabs have a cotton or a rayon tip that is made of fibres wrapped around a plastic rod, whereas synthetic swabs are made of various polymers, such as polyester, polyurethane or nylon. Also, some polyester and nylon swabs may be flocked. Cotton and rayon swabs tend to trap bacterial cells within the fibre matrix, thus hampering the release of the cells in the recovery; in addition, some impurities may be released (Bruijns et al., 2018). Moreover, synthetic swabs are preferable for molecular analyses, as plant DNA may be released from cellulose-based swabs, thus contaminating the extracted microbial nucleic acids (Table 2.2). The performance of synthetic swabs further depends on the properties of the polymeric matrix. For example, nylon flocked swabs improve cell release because of an increased capillary action (Dalmaso et al., 2008), while polyurethane swabs are well-suited for sampling porous surfaces (Bruijns et al., 2018). However, experimental data indicate that microbial adhesion strongly depends on the features of the surface being sampled (Cai et al., 2019) and on factors such as the presence of exopolysaccharides and the frequency and intensity of cleaning procedures (Araújo et al., 2010). Moreover, Motz et al. (2019) recently performed a systematic comparison between different types of swabs by sampling surfaces spiked with different bacterial species, chosen for their different adhesive capacity. They

demonstrated that swab mass and surface area have a greater influence than swab composition in retrieving microorganisms.

Nucleic acid extraction kits and protocols are also an important point to consider. Most commercial kits currently available are optimized for stool, foods or soil samples rather than for the extraction of microbial nucleic acids from low-biomass swab samples such as those from food processing environments. Besides having usually low microbial loads, these surfaces may be contaminated with detergents, disinfectants or residual food matrix materials that may inhibit subsequent enzymatic steps. For these reasons, the optimization of a microbial DNA extraction protocol for this specific type of samples is crucial.

The most recent innovations in HTS are the so-called "Third Generation Sequencing" technologies, which are based on the use of real-time, high throughput, and - in some cases - portable sequencers. These novel methods are more suitable than Next Generation Sequencing platforms for quick and on-site sequencing, providing longer reads than previous generation of sequencers (Midha et al., 2019). Reasonably, these high-throughput and portable sequencers could be soon used directly in factory sites for real-time monitoring of microbial communities.

Finally, once the DNA has been sequenced, bioinformatics and statistical skills are necessary for data analysis. Data analysis can be considered the real bottleneck in the routine application of HTS in the food industry, since personnel specialized in bioinformatics would be necessary. Indeed, novel data-scientist figures with a background in food microbiology would be important in helping food companies to get the most from metagenomics data and understand how to integrate and exploit these kinds of analysis in a quality and safety management plan. Therefore, innovative courses directed to understand the use of these novel techniques in food industries should be integrated in higher education institutions for all food science programs. In addition, events and demonstration activities for food business operators would be of utmost utility to achieve a successful knowledge and innovation transfer.

2.4. Microbiome mapping and EU regulation

According to EU regulation No 852/2004 on the hygiene of foodstuffs, the primary responsibility for food safety rests with the food business operators, who, following a preventive approach, should establish and operate food safety programmes and procedures based on the Hazards Analysis and Critical Control Points (HACCP) principles to ensure that food safety is not compromised. Validation and verification of HACCP procedures are accomplished through, among others, the compliance with microbiological criteria defining the acceptability of the processes and the end-products, which are defined under EU Regulation No 2073/2005. That piece of regulation highlights that sampling of the production and processing environment can be a useful tool to identify and prevent the presence of pathogenic microorganisms in foodstuffs and specifically mentions that food business operators manufacturing ready-to-eat foods shall sample the processing areas and equipment for *L. monocytogenes* and those manufacturing dried infant formulae or dried foods for special medical purposes intended for infants below six months for *Enterobacteriaceae* as part of their sampling schemes. All environmental sampling activities currently undertaken by food business operators are therefore based on tracing specific foodborne hazards and/or indicators using classic tools for the isolation and identification/confirmation of target microorganisms. These have numerous limitations, including the long time required to obtain results, which delays the implementation of corrective measures when problems are encountered. In addition, according to the EU Regulation, environmental sampling shall be performed following the ISO standard 18593 on horizontal methods for surface sampling as a reference. However, these standard methods have been developed for the specific aim of isolating and enumerating microorganisms from certain particular taxa. HTS-based approaches, given their properties highlighted in previous sections, have the potential to revolutionize the way food business operators approach environmental monitoring activities within their food safety management systems. However, the future transition from classical microbiological techniques to HTS-based microbiome monitoring techniques will require the development of new standards,

covering aspects from sampling to bioinformatic analyses and interpretation of results, specifically tailored to the needs of food business operators. These new standards should be robust and flexible to support the fast development of commercially available innovations, but also to leave space to account for rapid advances in technology allowing the necessary updates when methods become outdated. Moreover, they should be internationally agreed and validated on a global scale to provide evidence of their reproducibility and accuracy (EFSA, 2019). Nevertheless, in the long-term, the integration of HTS-based microbiome analysis in food safety policies will also require the translation of the complex outputs provided by metagenomic tools into quantifiable and easy to interpret microbiological process criteria allowing rapid decision making by the food industry.

2.5. Conclusions and future perspectives

The resident microbiome in food factories plays an important role in influencing food quality and safety. Production activities, environmental and process parameters shape the microbial communities inhabiting food facilities. Monitoring of the food industry environmental microbiome by up-to-date sequencing-based strategies is a promising tool that could support overall quality and safety management plans. However, despite the decreasing cost of these technologies, their implementation as routine practices with respect to the environmental monitoring in the food processing industry is still challenging. In this regard, the generation of results from broad and structured initiatives that include the development, validation and dissemination of microbiome mapping strategies can greatly assist the food industry and related stakeholders to adopt next generation procedures for their quality assessments and develop improved sustainable production chains to be better prepared for possible specific regulatory changes in the food sector.

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CHAPTER 3

Evidence of virulence and antibiotic resistance genes in minimally processed vegetables-producing facilities

3.1. Introduction

Fresh vegetables are an essential part of a healthy dietary pattern and have been used for centuries (Randhawa et al., 2015). Indeed, these foods contain high levels of phytochemicals, fiber and minerals (Liu, 2013). International organizations such as the World Health Organization (WHO) suggest a 400 g/day intake of vegetables (World Health Organization, 2020).

Although the consumption of raw vegetables is highly recommended, their use arises concerns about their safety. Indeed, raw vegetables are subjected to limited processing before their arrival to the shelf, that includes selection and (optional) portioning and removal of non-edible parts. In some cases, a rough washing step is applied. Therefore, they might represent a risk for the health of the consumers, since it has been demonstrated that several pathogenic taxa can survive and proliferate on their surfaces (Al-Kharousi et al., 2016; Tatsika et al., 2019; Yin et al., 2022). This evidence, together with the inefficiency of domestic washing procedures to remove microorganisms (Tatsika et al., 2019), should draw the attention of the food industry and consumers on the potential outcome that might derive from the consumption of contaminated products.

Recent reports (Carstens et al., 2019) indicate that a large part of foodborne outbreaks can be linked to the consumption of minimally processed vegetables such as sprouts, lettuce, cucumbers and spinaches, with a wide range of associated symptoms, including bloody diarrhea and gastroenteritis. Most of these outbreaks are attributed to well-known pathogens conveyed by fresh vegetables, such as *Salmonella enterica* and *Escherichia coli* O157:H7 (Carstens et al., 2019), although the range of hazardous microorganisms that could survive and replicate in fresh vegetables is wider, also including *B. cereus* and *Pseudomonas aeruginosa* (Afolabi et al., 2011;

Fiedler et al., 2019; Rosenquist et al., 2005; Yu et al., 2019). In addition, several opportunistic pathogens, such as *Pantoea agglomerans*, *Klebsiella pneumoniae* and *Rahnella aquatilis* have been also reported (Al-Kharousi et al., 2016).

Also, contamination of fresh vegetables might occur at multiple points from farm to fork. The soil is the primary source of pathogenic microorganisms, since minimally processed vegetables grow within or near the ground, although irrigation water, fertilizers and insects may also carry hazardous microbes (Carstens et al., 2019). However, post-harvesting and processing of vegetables also contribute to contamination, due to the contact with transportation vehicles, operators and equipment inhabited by pathogens (Carstens et al., 2019).

Multiple studies have shown that some pathogenic/commensal bacteria associated with food and its production environment may carry out Antibiotic Resistance Genes (ARGs) in their genomes, which might be transferred to other microorganisms through Mobile Genetic Elements (MGEs) and represent a potential hazard (Oniciuc et al., 2019). According to WHO, antibiotic resistance (AR) is one of the most important public concerns, since the overuse of antibiotics in all fields (e.g., agriculture, farming and individual medications) has led to the selection of resistant strains (Ventola, 2015; World Health Organization, 2020). Indeed, farm soils have been addressed as a "hot spot" of resistant microorganisms (Founou et al., 2016).

Food processing environments are an important reservoir of microorganisms that may be easily transferred to the product. Indeed, microbial consortia might adapt to the specific microclimatic conditions of the food processing plant and establish on the surfaces by forming biofilms (De Filippis et al., 2021). In such circumstances, bacteria might resist to cleaning and disinfection procedures, becoming resident in the food processing environment. For example, *Salmonella* and *Acinetobacter* isolated from vegetables can produce biofilms on various types of surfaces (Bae et al., 2014; Isoken, 2015). Indeed, the combination of AR and biofilm formation represents a successful microbial strategy to promote the survival under environmental stress conditions

(Carter & Brandl, 2015; Xu et al., 2021) and enhance the long-term colonization of environmental surfaces associated to food production.

Few investigations about the colonization of the fresh vegetables handling environment by bacteria and on the assessment of their resistance to antimicrobials are available. This topic needs attention and proper investigation, since AR microorganisms embedded into biofilms on industrial surfaces might end up on the vegetables, which are often consumed without prior cooking, spreading ARGs and representing a safety hazard.

The purpose of this work is to assess the taxonomic composition, the antimicrobial resistance and virulence potential (including genes involved in biofilm formation) of the microbiome residing in the environment of three facilities producing minimally processed vegetables in order to ascertain the relevance of the environmental microbiome on the safety of the end products.

3.2. Materials and methods

3.2.1. Samples collection, DNA extraction and whole metagenome sequencing

Three facilities from Southern Italy producing minimally-processed vegetables (named G, J, P) were visited (February–October 2020) after the completion of the routinary cleaning procedures. Facility G produced spinaches (*Spinacia oleracea*), whereas facilities J and P produced endive (*Cichorium endivia*) and arugula (*Eruca vesicaria*), respectively. Raw vegetables were not subjected to prior washing, but the process included three steps: separation of soil particles from leaves (by vibration/optical sorting), portioning and packing. Prior to the sampling, details about the cleaning and sanitation procedures were recorded (Table 3.1).

Table 3.1 Description of the sanitation procedure adopted by each facility. DFC = Disinfectant concentration; SF = Sanitation frequency, R1 and R2 = Rinsing.

Facility	Detergent	R1	Disinfectant	DFC – CT (min)	R2	SF
G	Pressurized air/water	H_2O 60°C	Sodium hypochlorite	25 mL/L – 10 min	H_2O 65°C	Weekly
J	Pressurized air	NA	Sodium hypochlorite	NA	25°C	Weekly
P	Pressurized air	NA	Sodium hypochlorite	10 mL/L – 5 min	25°C	Weekly

Food contact (FC) and non-food contact (NFC) surfaces from the facilities were sampled using Whirl-Pak Hydrated PolyProbe swabs (Whirl-Pak, Madison, Wisconsin, US), covering an area of about 1 m^2, or a sampling unit (e.g., one knife, one box). In addition, swabs were collected from hands/aprons of employees working on the sampled production line. Five swabs from each sampling point were collected and pooled before DNA extraction. A total of 32 pooled composite samples were available from the three facilities, including vegetables (about 100 g) at the beginning (n = 6) and at the end of the processing (n = 6), environmental FC swabs (n = 12) and NFC swabs (n = 5), and swabs from hands/aprons of employees (n = 3).

All the samples were stored at 4 °C and transported to the laboratory, where they were pre-processed within 2 h.

In the laboratory, the 5 swabs from each surface were pooled together, and 10 mL of Phosphate Buffered Saline (PBS) 1X were added. In addition, the surfaces of the raw materials and final products were swabbed with 5 swabs/sample in sterile conditions and the five swabs per sample were pooled together and processed following the same procedures as for the environmental swabs. Microbial cells were detached from the pools of swabs using a Stomacher (300 rpm × 30 s), then the supernatant was collected and aliquoted in 5 mL sterile tubes (Eppendorf, Hamburg, Germany). The tubes were centrifuged at 14.000 × g for 2 min, then the cellular pellet was washed twice with 2 mL of sterile PBS. The cellular pellets were stored at −80 °C until further processing. DNA extraction was performed from the pellets using the PowerSoil Pro Kit, adopting a modified version of the standard protocol previously validated to increase the total microbial DNA yield from food processing environments (Barcenilla et al., under review). Briefly, these modifications were the use of Qiagen's UCP MinElute Spin Columns instead of the standard spin columns; addition of 600 µl 100 % isopropanol to the silica columns during DNA binding step; addition of 40 % EtOH (100 %) to solution C5 on wash step; and perform the final elution in a volume of 20 µl. Then, the concentration of extracted DNA was quantified using the Qubit HS Assay (Thermo Fisher Scientific, Waltham, Massachusetts, United States).

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

3.2.2. Bioinformatic and statistical analysis

Reads were quality-checked by PRINSEQ lite (version 0.20.4; Schmieder & Edwards, 2011) using parameters "-trim_qual_right 5" and "-min_len 60", then taxonomic profiles were obtained using Kraken2 (Wood et al., 2019), jointly with the "maxikraken2" database (available at https://lomanlab.github.io/mockcommunity/mc_databases.html), using default parameters. Bacterial counts were extracted from each profile and merged in one file using an in-house script, then the proportion of reads mapping to each taxon was computed. In addition, SourceTracker2 (Knights et al., 2011) was used on the bacterial counts, with the options "–beta 0", "–source_rarefaction_depth 1000", "–sink_rarfaction_depth 1000" and "–burnin 500". For this analysis, the initial product and the surfaces were defined as "source", whereas final products were labelled as "sinks".

For each sample, reads were independently assembled into contigs using MegaHIT (version 1.2.2; Li et al., 2016), filtering out contigs shorter than 1,000 bp. Then the reads from each sample were mapped to the corresponding sample contigs using bowtie2 (version 2.2.9; Langmead & Salzberg, 2012), with parameters "–very-sensitive-local" and "–no-unal". The *jgi_summarize_bam_contig_depths* script, from MetaBAT v2.12.1 (Kang et al., 2015), was used to calculate contigs depth values from the sam files obtained by bowtie2 alignment, mandatory for per- sample contig binning by MetaBAT in order to reconstruct Metagenome-Assembled Genomes (MAGs). Only contigs longer than 1,500 bp were binned.

The CheckM "lineage_wf" workflow (version 1.0.13, Parks et al., 2015), was used to assess the quality of MAGs, and only those with completeness ≥ 50 % and contamination < 5 % (i.e., medium/high quality MAGs, with high quality MAGs being those with completeness > 90 %; Pasolli et al., 2019) were retained for further analyses.

Pairwise Mash distances (version 2.0; option "-s 10000"; Ondov et al., 2016) were computed between the MAGs, and a 5 % dissimilarity threshold was used to assign MAGs to a Species-level Genome Bin (SGB), as previously suggested (Pasolli et al., 2019). Taxonomy was inferred by comparing the most complete and less contaminated MAG from each SGB to the MetaRefSGB database (December 2020 release; Pasolli et al., 2019), selecting 5 %, 15 % and 30 % dissimilarity threshold for species, genus and family level, respectively.

In addition, phylogeny of MAGs was inferred with the tool GT-DBTk (version 0.3.3; Chaumeil et al., 2020) using the "classify_wf" and "infer" commands, and the resulting tree was visualized in iTol (version 6.5.3; Letunic & Bork, 2021).

In order to assess the pathogenetic potential of 4 MAGs taxonomically assigned to *B. cereus* sensu stricto, we manually downloaded the sequences of *hblCDA*, *nheABC*, *cytK* and *entFM* operons from the NCBI GenBank database. These genes are responsible for the secretion of *B. cereus* enterotoxins (Senesi & Ghelardi, 2010). Genes were predicted from MAGs using Prokka (version 1.11; Seemann, 2014), then they were mapped to the previously collected sequences using blastn (version 2.2.30; options "-evalue 0.00001", "-perc_identity 50" and "-word_size 7").

Metagenome assemblies were screened for AR and Virulence Factor (VF) genes using TORMES (version 1.3.0, Quijada et al., 2019). Only contigs matching with identity and coverage ≥ 80 % were retained for further analyses. Contigs were taxonomically classified with Kraken2 as previously described, then Platon (Schwengers et al., 2020) and PlasFlow ("threshold 0.8"; Krawczyk et al., 2018) were used to assess whether ARG-associated contigs were part of plasmids or chromosomes. In addition, reads per kilobase per million reads (RPKM) abundance of both AR and VF contigs was estimated by multiplying the number of reads mapping to each gene for 10^9 and normalizing for gene length and total number of bacterial reads in the metagenome.

Data visualization and statistical analysis were performed in R environment (version 4.1.3; https://www.r-project.org). Mean values for each group were compared using the Wilcoxon rank sum test ("wilcox.test" from "base" package), with a 0.05 p-value threshold for significant results

(unless otherwise stated). The functions “vegdist” and “diversity” from the “vegan” package were used to compute Bray-Curtis distances and alpha diversity indices, respectively, whereas “geom_point” from “ggplot2” plotted the first two Principal Coordinates. Barplots figures were produced using “geom_col” from the “ggplot2” package.

3.2.3. Data availability

Raw reads are available on the Sequence Read Archive of the National Center of Biotechnology Information (NCBI) under the accession number PRJNA897099.

3.3. Results

3.3.1. Taxonomic composition of the microbiome of raw materials, end products and environments and SourceTracker analysis

Pseudomonas was the most abundant taxon in both vegetables and surfaces, with a mean percentage of reads of 16.44 ± 10.14 % and 8.02 ± 20.16 %, respectively, followed by *Bacillus* (7.53 ± 22.58 % and 5.29 ± 13.27 %). Other abundant genera were *Kocuria* and *Acinetobacter*, which reached 4.77 ± 5.28 % and 4.55 ± 14.63 % on surfaces, respectively. In addition, remarkable differences in taxonomic composition were observed between FC/NFC surfaces and food products, as showed by a PCoA based on the Bray-Curtis distance (adonis $p < 0.001$, Figure 3.1). This separation might be partially explained by *Pantoea*, *Pseudomonas*, *Enterococcus* and *Escherichia*, that were significantly more abundant on vegetables (both at the beginning and at the end of the processing), as well as *Paracoccus* and *Actinomyces*, that were more abundant on surfaces (both FC and NFC). However, no clear separation of FC and NFC surfaces was observed (Fig. 3.1). No significant differences were found in alpha diversity parameters among the sample groups.

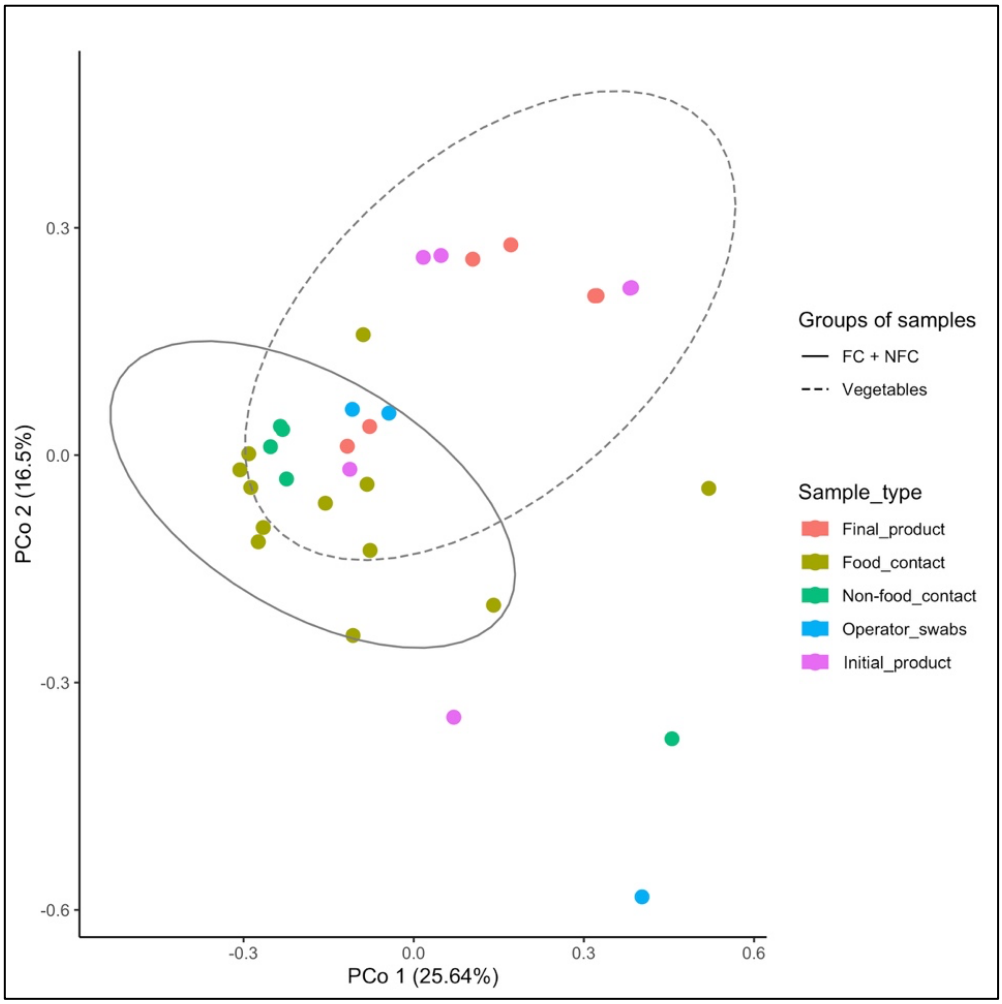

Figure 3.1 PCoA based on the Bray-Curtis distance performed on the genus-level bacterial profiles obtained with Kraken2. Points are color-coded according to the sample type. Ellipses are drawn around surfaces (FC + NFC) and Vegetables (Initial + Final products).

The analysis with SourceTracker2 identified the initial vegetables as the major source contributor to the microbial composition of the final products. However, FC/NFC surfaces in the production area also had a leading role for all the three facilities, with the overall contribution ranging between 10.0 and 39.2 % (Fig. 3.2). Moreover, there was a high contribution estimated from unknown sources (i.e., potential sources of contamination that we did not sample), which ranged between 21.2 and 34.7 % in the different facilities (Fig. 3.2).

3.3.2. MAGs reconstruction and phylogenetic analysis

Overall, a total of 290 medium/high quality bins were reconstructed from the metagenomes. Of these, 181 were included into SGBs with > 1 MAG. From the phylogenetic analysis of MAGs, a separation between foods and surfaces emerged (Fig. 3.3): in particular, vegetables were

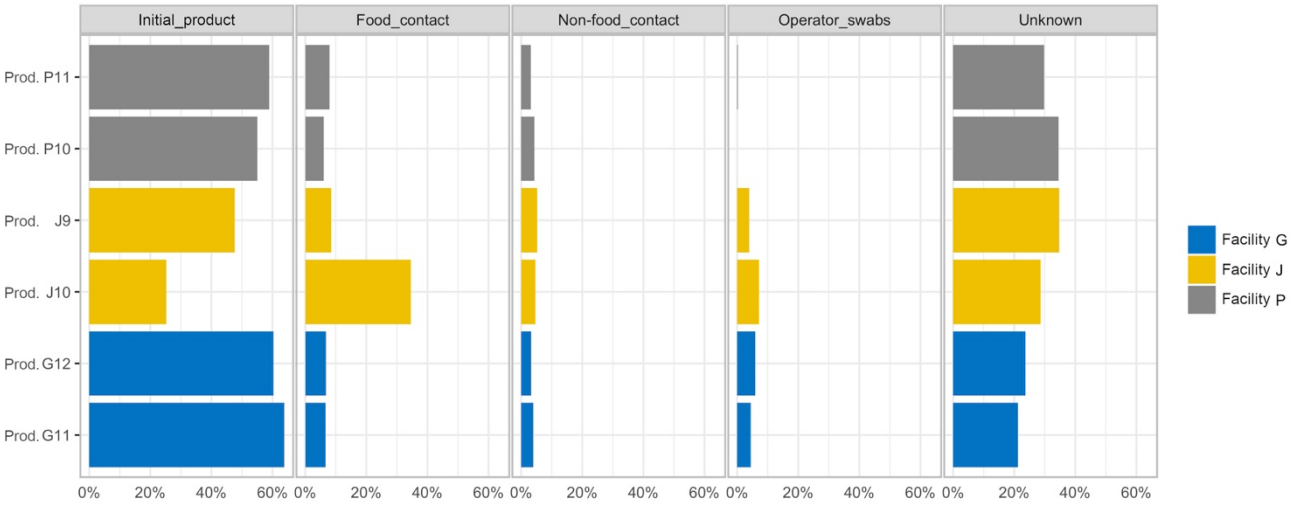

Figure 3.2 Barplot showing the percentage contribution (x-axis) of each source of contamination to the taxonomic composition of the final products (y-axis). The 2 final products from each facility were analysed independently.

Figure 3.3 Phylogenetic tree of all the medium/high quality MAGs reconstructed from the metagenomes.

dominated by *Proteobacteria*, with genomes assigned to *Pantoea* (n = 9), *Xanthomonas* (n = 4), *Psychrobacter* (n = 5), *Pseudomonas* (n = 7) and *Acinetobacter* (n = 7), whereas *Actinobacteria*

(*Kocuria*, n = 27; *Glutamicibacter*, n = 6) and *Bacillota* (*Bacillus*, n = 8; *Staphylococcus*, n = 5) were more prevalent on surfaces.

In addition, 4 out of 8 genomes assigned to the *Bacillus* genus were highly similar to *B. cereus*, a well characterized human pathogen (Fig. 3.4). Three of these MAGs were reconstructed from 3 FC surfaces from facility "G", whereas 1 was from the operator's hands from facility "J". The alignment of genes predicted from *B. cereus* MAGs to the characteristic virulence gene sequences from this taxon (i.e., *hblCDA*, *nheABC*, *cytK* and *entFM*) suggests the presence of the pathogenic operons in the genomes reconstructed from the surfaces.

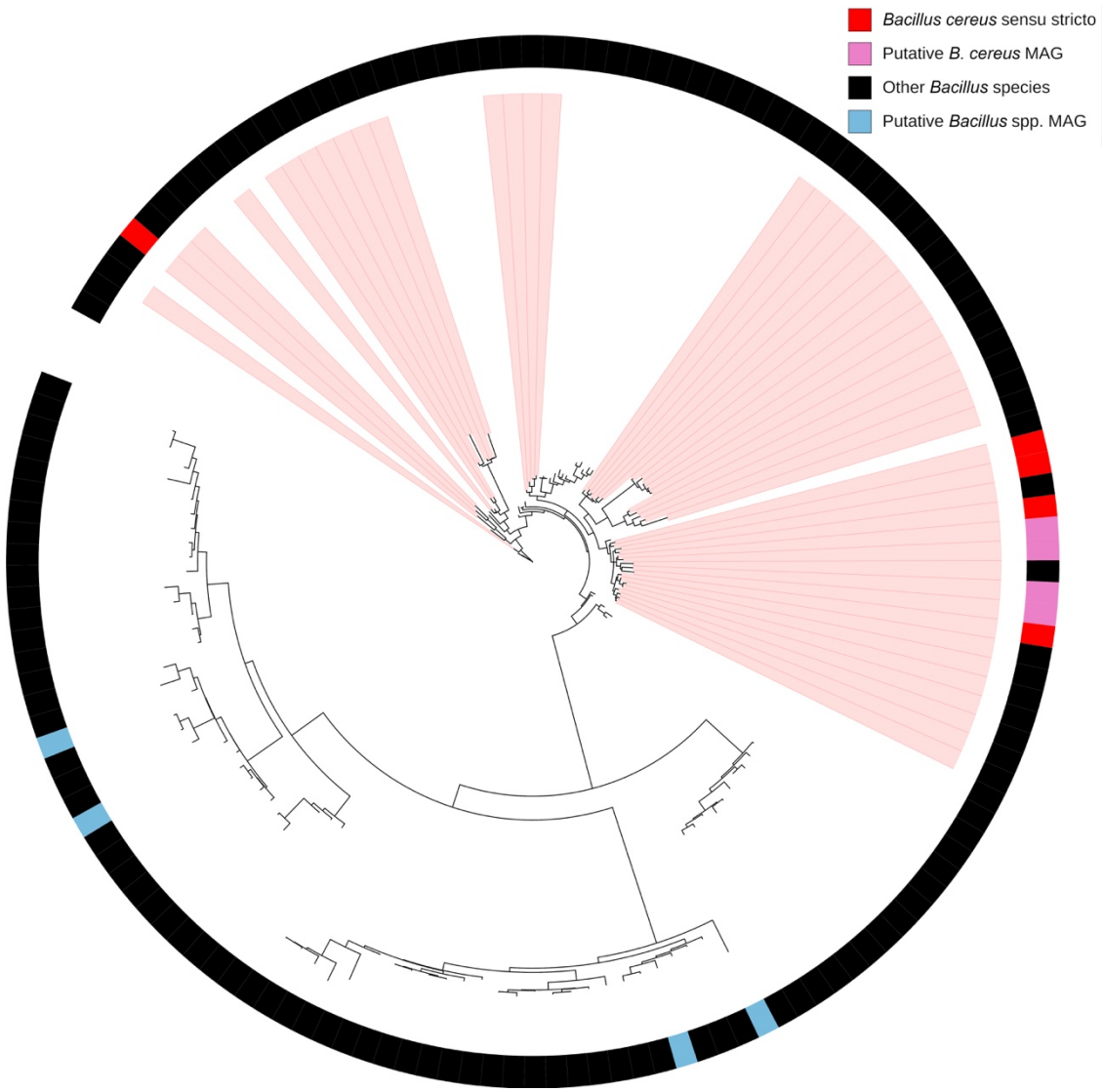

Figure 3.4 Phylogenetic tree of a subset of NCBI RefSeq genomes spanning across multiple *Bacillus* species and those MAGs from surfaces and vegetables attributed to *Bacillus*. Clades highlighted in red belong to the *Bacillus cereus* group.

3.3.3. Several taxa from environmental surfaces and vegetables carry ARGs

The screening of the metagenome assemblies for the presence of ARGs highlighted that 277 contigs carried at least one ARG. According to the Kraken2 taxonomic assignment, *Bacillus* harboured the highest number of AR related contigs, with 45 contigs carrying ARGs. Of these, 19 were assigned to *B. cereus*, 7 to *B. clausii* and 6 to *B. thuringiensis*. In addition, *Pseudomonas*, *Pantoea* and *Acinetobacter* contributed significantly to AR, with 30, 22 and 20 contigs, respectively. *Bacillus* showed a high number of AR genes from the beta-lactams (n = 20), fosfomycin (n = 6) and multidrug (n = 6) antimicrobial classes, and notably, 8 contigs carried genes related to resistance to Critically Important Antibiotics (CIA), as described by the World Health Organization (World Health Organization, 2018). On the oppo- site, contigs associated with *Pseudomonas* showed multidrug resistance genes (n = 27), but none of them was related to CIA. Regardless of the taxonomic assignment, FC surfaces hosted the highest number of AR-related contigs, with an average of 11.9 contigs per sample, compared with NFC surfaces (avg. 6 contigs/sample), samples from operators (avg. 6.6 per sample), and vegetables at the starting (avg. 5.6 per sample) and ending point (avg. 8.1 per sample) of the process. In addition, 42 out of 143 AR-associated contigs recovered from FC surfaces might be part of plasmids, which were mainly linked to *Acinetobacter*, *Bacillus* and *Staphylococcus*.

In addition, the abundance of AR-associated contigs was estimated. Overall, genes showing resistance to tetracyclines were the most abundant, with a mean RPKM value of 122.9 ± 150.3, followed by genes associated with resistance to multiple drugs (96.1 ± 243.4), macrolides (83.3 ± 143.2) and streptomycin (70.7. ± 28.2). Interestingly, 16 out of the 36 most abundant ARGs (i.e., with RPKM > 50) coded for resistance to multiple drugs and were assigned to *Bacillus* sand *Pseudomonas* spp.

Abundance estimation of ARGs further showed that FC/NFC surfaces have a leading role in the potential transfer of ARGs to the products, since no significant differences were observed between surfaces and vegetables (data not shown). *Bacillus*, *Acinetobacter*, *Staphylococcus* and

Pseudomonas contributed the most to AR on surfaces (Fig. 5). Also, FC surfaces hosted a broader range of ARG classes, some of which were totally absent from other sample groups (e.g., streptomycin, streptogramin; Fig. 3.5). Finally, there were no significant differences in AR abundance among the three facilities.

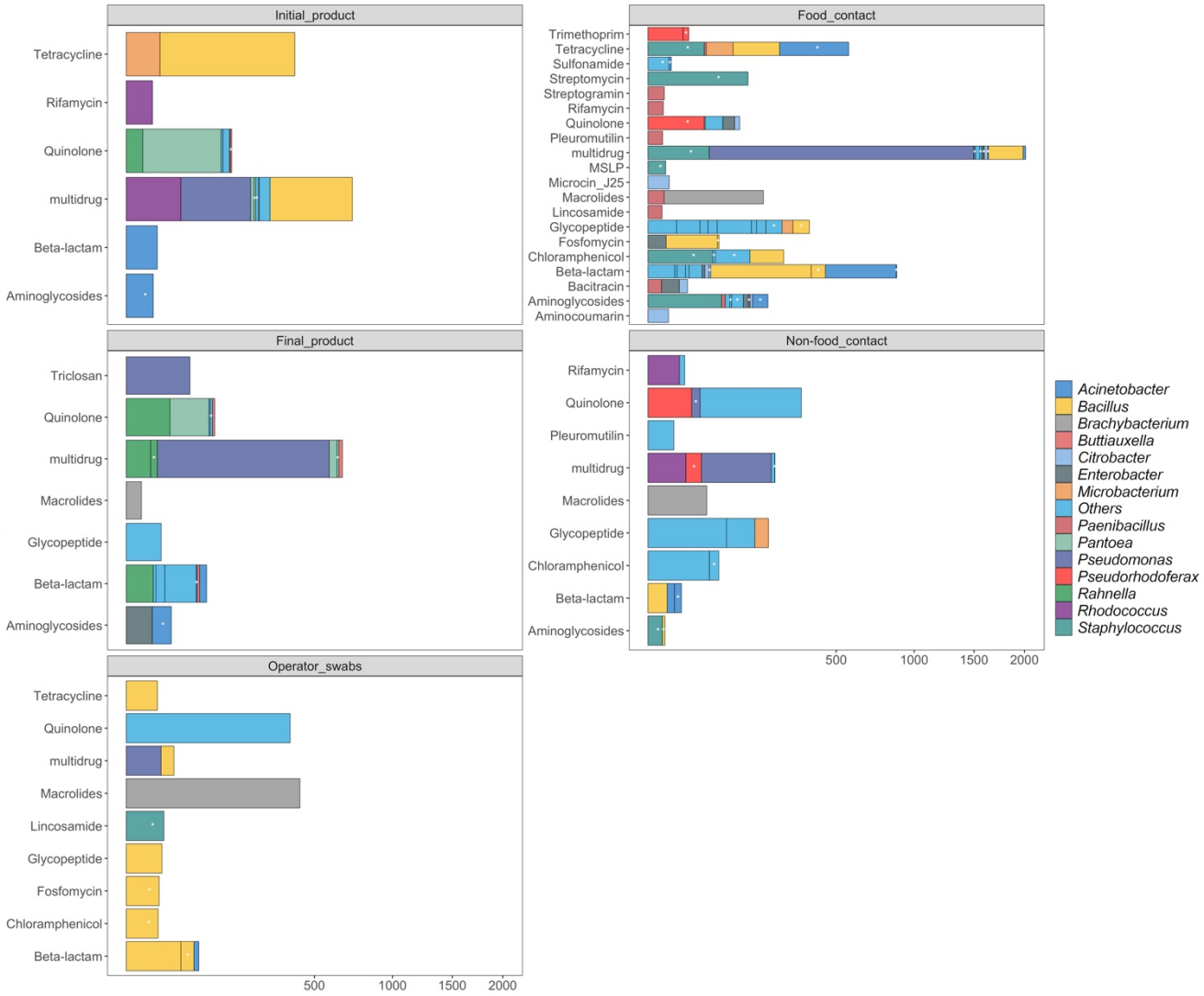

Figure 3.5 Barplot showing, for each sample category, the abundance in RPKM of the Antibiotic Resistance Genes classes. Bars are color-coded according to the taxonomic assignment of the ARG-carrying contigs reported by Kraken2. Genes marked with an asterisk (*) are reported to be part of plasmids according to Platon and/or PlasFlow.

3.3.4. Pseudomonas virulence factors are widespread on surfaces and vegetables

We used the same approach to estimate the abundance and assess the taxonomic assignment of genes coding for Virulence Factors (VFs). Overall, 658 contigs carrying VFs were found in the

metagenomes, 504 of those were assigned to the genus *Pseudomonas*, while 33, 23 and 11 belonged to *Bacillus*, *Rhizobium* and *Pantoea*, respectively. In addition, vegetables (both at starting and ending point of the process) reported the highest count of VFs. Contigs related to motility were the most widespread on vegetables, as well as on FC surfaces. On the contrary, 17 and 12 contigs out of 32 associated with exotoxin production were reconstructed from FCS and operator swabs, respectively. Interestingly, all except one of these contigs belonged to *Bacillus*. Finally, abundance analysis showed that "Biofilm", "Effector delivery system", "Immune modulation" and "LPS" VF classes did not differ significantly between surfaces and vegetables from all the facilities (Fig. 3.6).

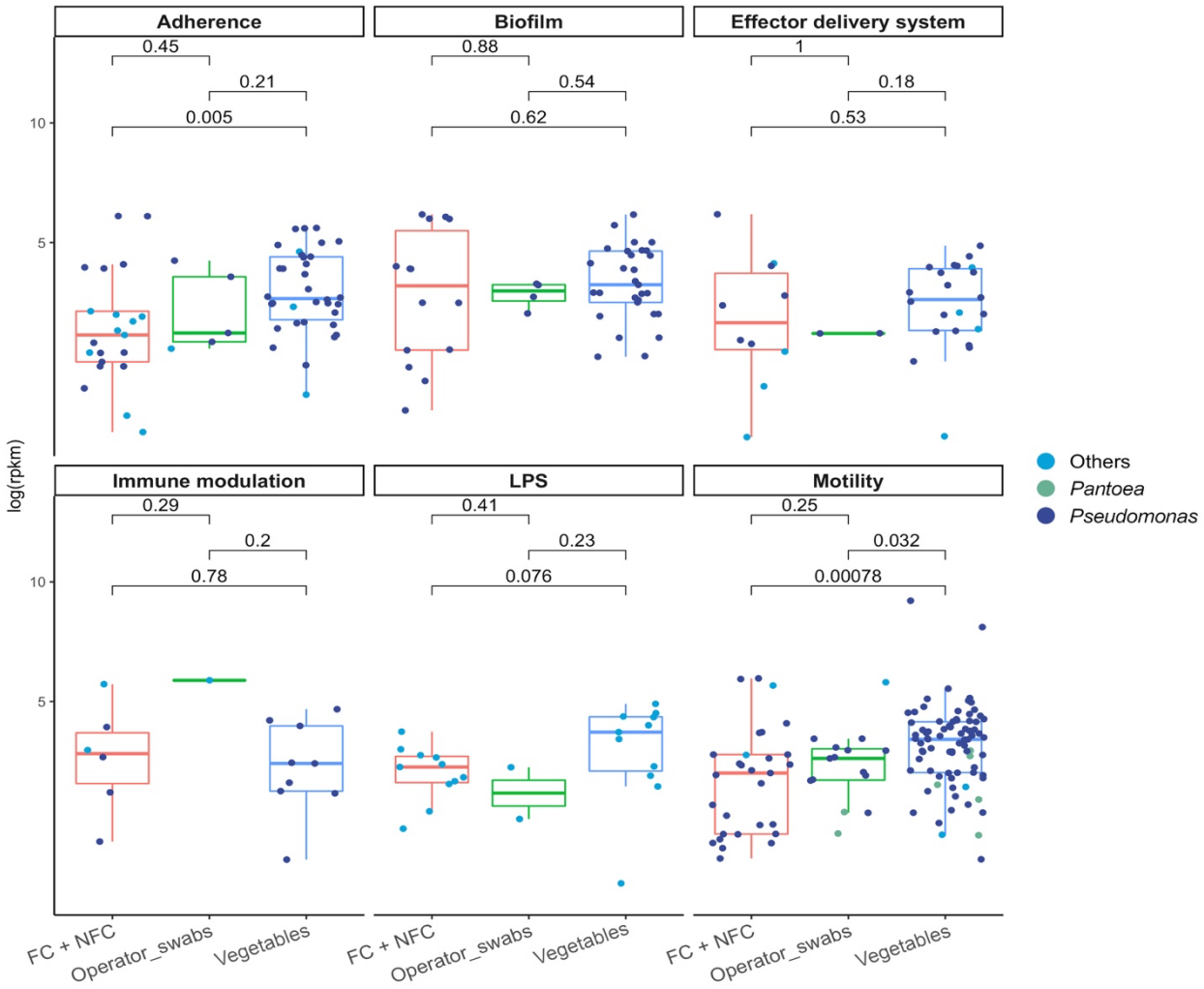

Figure 3.6 Boxplot showing the RPKM abundance (in log scale) of several Virulence Factor Genes (VFGs) for each group of samples ("FC+NFC", "Operator swabs" and "Vegetables"). Points are color-coded according to the taxonomic assignment of the VFG-carrying contigs reported by Kraken2.

3.4. Discussion

The environmental microbiome of vegetable processing plants can be an important factor influencing the quality and safety of the final product. Therefore, the taxonomic composition and potential genomic features of the microbiome need in depth investigations. The microbial composition of vegetables was largely consistent with previously published reports. Indeed, *Pseudomonas*, *Bacillus* and *Pantoea* were previously identified as the core microbiota of fruit and green leafy vegetables (Sequino et al., 2022; Soto-Giron et al., 2021; Taffner et al., 2020). Most of the highly abundant taxa identified in this study are common soil inhabitants (Deakin et al., 2018; Jiao et al., 2019; Simonin et al., 2022), mainly belonging to the *Proteobacteria* phylum, which is generally related to carbon, nitrogen and sulphur cycling (Mhete et al., 2020).

In addition, vegetables and surfaces harbour different microbial communities, as detected at read-level analysis, which was further confirmed by the taxonomic identification of MAGs that showed surfaces as dominated by *Bacillota* and *Actinobacteria*, while *Proteobacteria* and *Bacteroidota* were more prevalent on vegetables. Although varying in composition, both vegetables and surfaces host a high number of microbial taxa. Indeed, alpha diversity indices showed no difference between foods and clean surfaces, suggesting that the stressful environmental conditions (i.e., the sanitation procedure) might not be able to alter the persistence of a highly diverse microbiome on sanitized surfaces, as previously reported (Møretrø & Langsrud, 2017). Also, we observed a range of potential virulence factors, a wide range of molecules and cellular structures produced by pathogenic microorganisms to help overcoming host's defence systems and cause disease (Chen et al., 2005; Leitão, 2020), which mainly belonged to *Pseudomonas* sand were related to bacterial adherence, biofilm production and effector delivery systems, also linked to the production of biofilm in *Pseudomonas* (Chen et al., 2015). Such genes reached a high abundance in the food production environments (Figure 3.6). *Pseudomonas* have been widely reported as common inhabitants of food-handling environments (De Filippis et al., 2021; Sequino et al., 2022; Stellato et al., 2016). Their adaptation to environmental stress through the production

of biofilms has been widely described, especially for *P. aeruginosa* (Pericolini et al., 2018), even though it has been observed that this ability is common within the genus (Fazli et al., 2014; Mann & Wozniak, 2012). In addition, biofilms produced by *Pseudomonas* may potentially entrap pathogenic microbes, thus protecting them from external stress (Guzmán et al., 2020). Evidence suggests that *Pseudomonas* are often present in multi-species biofilms involving pathogenic bacteria (Quintieri et al., 2021), and the non-pathogenic species *P. fluorescens* is able to enhance the adhesion and biofilm formation of *Listeria monocytogenes* (Maggio et al., 2021; Puga et al., 2018).

Moreover, the extracellular polymeric substance (EPS) that protects cells embedded into biofilms, also limits the entry of biocides such as disinfectants, exposing microorganisms to sub-Minimal Inhibitory Concentrations (MIC) of these compounds (Flores-Vargas et al., 2021). It has been shown that exposition of some bacterial strains to sub-MIC of quaternary ammonium compounds and sodium hypochlorite – two of the most used disinfectants in the food industry – might enhance the acquisition of resistance to fluoroquinolone, beta-lactam and amino-glycoside antibiotic classes (Nasr et al., 2018; Oniciuc et al., 2019; Piovesan Pereira et al., 2021), as a result of cellular response mechanisms that strengthen the tolerance of microorganisms to multiple biocide agents (i.e., cross-resistance; Wales & Davies, 2015). This phenomenon, together with the natural AR pattern occurring in soil and vegetables (Wang et al., 2022), might explain the broad diversity and high abundance of ARGs from different taxa (including *Bacillus* and *Acinetobacter*) that we observed on sanitized FC surfaces (Figure 3.5), as well as the presence of toxigenic *B. cereus* strains on some of these surfaces.

Sanitation of food processing plants is extremely important to avoid foodborne outbreaks, especially in facilities producing fresh vegetables, where the absence of lethal operation units promotes the survival and growth of pathogens (2008). Nonetheless, the so-called "disinfectant-induced antibiotic resistance" (Chen et al., 2021) might have a negative outcome on the consumer's health (Jin et al., 2020). Stakeholders should seriously address this problem,

promoting the use of alternative compounds in order to limit the long-term spread of ARGs (Tarricone et al., 2020).

We were able to reconstruct 9 medium/high quality MAGs belonging to *Pantoea agglomerans* from both initial and final products. According to some reports, this genus was sporadically isolated from nosocomial environments, and may be implicated in infections, specifically in immunocompromised patients (Walterson & Stavrinides, 2015). Also, the biofilm formation ability (Yannarell et al., 2019) and the antibiotic resistance (Guevarra et al., 2021) of *P. agglomerans* have been discussed. Consistently with results from Guevarra et al. (2021), we found a high abundance of *Pantoea* contigs coding for resistance to quinolones and multiple drugs, mainly distributed in vegetables.

We attempted to identify the sources of contamination determining the taxonomic composition of the final product. Results from this analysis suggest that the microbiome of the vegetables at the end of the process mostly reflect that of initial vegetables. This was not surprising, since none of the processing steps strongly influences the structure or the properties of vegetables. However, despite the short contact time of vegetables with surfaces, an important influence of FCS on the microbial composition of the final product was observed in all the three facilities. This suggests that taxa from surfaces might end up in the final product, potentially reaching the gut after ingestion, since this product is commonly consumed raw.

Notably, several of the AR genes that we found across all the samples were associated with mobile elements, hence they might be transmitted to human pathogens. Previous reports already suggested that vegetables and minimally processed foods contribute the most to shape the gut resistome (da Silva et al., 2021), and HGT events involving bacteria from vegetables (mostly *Proteobacteria*) and from the gut microbiome have been documented (Blau et al., 2018; Ghaly et al., 2017).

In conclusion, we showed that sanitation procedures in minimally processed vegetables producing facilities might be ineffective in eradicating hazardous microorganisms (such as *B. cereus*) from FCS, which also show a broad pattern of resistance to antibiotics. On the contrary, our data suggest

that the extensive use of biocides might exacerbate AR selection. Overall, our findings evidence that there is a need to integrate microbiome-mapping in food processing environments into the routine monitoring procedures applied in the food industry to support appropriate strategies for the safety of the products. Integration of microbiome mapping in food manufactures, together with compliance to good hygiene practices in harvesting and processing of vegetables, might help food business operators to ensure safety and quality of foods.

3.5. References

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CHAPTER 4

Psychrotrophic bacteria in an ice-cream manufacture environment

4.1. Introduction

Food contact surfaces comprise all the surfaces and tools that might be in contact with ingredients, production intermediates and final products throughout the whole food chain (Skåra & Rosnes, 2016). Food industrial surfaces might be home to a plethora of microorganisms, even after cleaning and disinfection (De Filippis et al., 2021), and in some cases, it has been suggested that the extensive use of disinfectants and antimicrobials might link to the tolerance of these microorganisms in food industrial environments (Valentino et al., 2022; Piovesan Pereira et al., 2021).

Also, potential negative outcomes of biocides-resistant microorganisms in food industries have been described. These microorganisms might produce biofilms, potentially incorporating and protecting pathogenic species which might be in turn involved in cross-contamination events and foodborne outbreaks (Carrascosa et al., 2021). In addition, even non-pathogenic species embedded in biofilms might become hazardous by acquiring antimicrobial resistance through horizontal gene transfer (Abebe, 2020).

Several studies tried to assess the transmission routes from contaminated surfaces to food products. For example, Buchholz and colleagues (2012) inoculated food contact surfaces and equipment with *Escherichia coli* O157:H7, showing that it could reach the final products with high levels. These results suggest that the food production environment might represent a primary source of the communities inhabiting the food products. However, survival of microorganisms transferred from surfaces to food products in the latter depends on several factors, such as the nutritional value of the product, as well as its storage temperature (Siroli et al., 2017). In this respect, ice creams deserve an insight. Ice creams are frozen dairy desserts widely consumed worldwide, consisting of a complex matrix made of ice crystals, air bubbles, fat globules, milk proteins and sugars.

Although frozen, these desserts might be particularly hazardous for immunocompromised people and children, since low contaminating loads of psychrotrophic pathogens such as *Listeria monocytogenes* and *Staphylococcus* spp. might be of concern (Nalbone et al., 2022; Pouillot et al., 2016). Indeed, in 2020 a batch of ice creams tested positive for staphylococci and staphylococcal enterotoxins, according to a report from EFSA. In addition, recent evidence suggests that antibiotic-resistant species might survive in ice creams, potentially reaching the human gut (Zhang et al., 2022; Sohel et al., 2022). However, to the best of our knowledge, no information about ice creams wastes due to spoilage microorganisms are available.

To date, only a few studies linking the microbial communities of the food processing environment to that of ice creams have been performed (Inuwa et al., 2017). Therefore, the purpose of this work is to taxonomically and functionally describe the communities residing in an ice creams food processing plant by sequencing of both V3-V4 region of the 16S rRNA gene PCR products and whole metagenome, with a major insight into the virulence and antimicrobial potential.

4.2. Materials and methods

4.2.1. Samples collection, DNA extraction, PCR amplification and sequencing

One facility producing industrial ice-creams was visited in July 2021. The food production environment was sampled after the cleaning and sanitation procedures, and before the next production shift.

Two different production lines were sampled, hereafter called “Line C” and “Line M”. Each surface was sampled by swabbing with 5 Whirl-Pak Hydrated PolyProbe swabs (Whirl-Pak, Madison, Wisconsin, US), as described before (see par. 3.2).

All the samples were transported in the laboratory and pre-processed within 24 hours. Swabs were processed as described in par. 3.2.

The total microbial DNA was extracted as previously described (see par. 3.2). The extracted DNA was then quantified using both the Qubit HS assay and the NanoDrop 3000.

One aliquot of the DNA (about 50 ng per sample) was used as a template for the PCR amplification of the V3-V4 hypervariable region of the rRNA 16S gene. Primers S-D-Bact-0341F5-CCTACGGGNGGCWGCAG and S-D-Bact-0785R5-GACTACHVGGGTATCTAATCC were used, and amplification cycles and reagent concentration have been previously reported (Quast et al., 2013). Library multiplexing and pooling were carried out according to the Illumina 16S metagenomic sequencing library preparation protocol using a Hamilton workstation, whereas the sequencing was performed on an Illumina MiSeq platform and using the MiSeq Reagent kit v2, yielding 2x250 bp reads.

Whole metagenome sequencing was carried out on the remaining part of DNA through an Illumina HiSeq platform.

4.2.2. bioinformatics analysis of 16S rRNA data

Forward and reverse raw reads were joined by FLASh (Magoč & Salzbeerg, 2011), then sequences were trimmed at the first instance of a base with a PHRED score < 20, and those that were shorter than 300 bp were discarded using PRINSEQ lite (Schmieder & Edwards, 2011). The remaining high-quality reads were imported into QIIME 1.9.1 (Caporaso et al., 2010) for following analysis. Briefly, OTUs were de-novo picked at 97% of similarity and representative sequences were mapped against the Greengenes 13_8 database using the RDP classifier (Wang et al., 2007). OTUs represented by a single sequence were discarded, and samples were rarefied at the same number of reads. Furthermore, the OTU table was collapsed at genus level and imported into R for statistical analysis.

4.2.3. bioinformatics analysis of whole metagenome sequencing data

Reads from the whole metagenomic sequencing were processed with PRINSEQ lite in order to trim low quality sequences and discard those being shorter than 60 base pairs. Furthermore, MegaHIT (version 1.2.2, Li et al., 2016) was used to assemble the metagenomes independently. Only contigs longer than 1000 bp were used for further analyses.

TORMES (version 1.3.0; Quijada et al., 2019) was used to screen for antimicrobial resistance and virulence genes in metagenomes. TORMES was used with the CARD (Alcock et al., 2020), ResFinder (Florensa et al., 2022) and ARGannot (Gupta et al., 2019) databases to search for antimicrobial resistance genes, whereas information about the virulence genes were retrieved by comparing the contig-predicted genes with the Virulence Factor Database (VFDB; Liu et al., 2022). Binning of contigs was performed with MetaBAT 2 (version 2.12.1, Kang et al., 2019), and quality of bins was assessed with CheckM (version 1.0.13, Parks et al., 2015). High quality bins (i.e., those with completeness > 90% and contamination < 10%) were clustered into Species-level genome bins (SGBs), choosing a MASH (version 2.0; Ondov et al., 2016) distance of 0.05 as threshold for species-level, as previously reported (Pasolli et al., 2019). Therefore, taxonomy was assigned to each SGB by comparing the best bin from each cluster to an annotated database.

4.2.4. Plotting and statistical analysis

All the plots and statistical analyses were performed in a R environment (https://www.r-project.org, version 4.1.3). Barplots, boxplots and scatter plots were produced using the 'ggplot2' R package (functions 'geom_col', 'geom_boxplot' and 'geom_point', respectively), whereas the plot of the genes coordinates was produced through the 'gggenes' package. The Wilcoxon's rank sum test ('wilcox.test' function from the 'stats' package) was used to assess significant differences between the groups, and a p-value of 0.05 was used as a threshold, unless otherwise stated. The principal coordinate analyses were conducted using the function 'cmdscale' from the 'stats' package, and the Bray-Curtis metric was used to compute pairwise distances.

4.3. Results

4.3.1. Taxonomic analysis

According to the 16S rRNA-based analysis, 976 taxa were found in 37 samples, although with a different relative abundance. Among these, *Pseudomonas* was the one with the highest average relative abundance (8.72 ± 4.72 %), followed by *Acinetobacter* (8.52 ± 3.20 %), *Rothia* (5.87 ± 1.63 %) and *Curvibacter* (5.12 ± 2.28 %). However, several differences were highlighted between

the two production lines, as well as between FC and NFC surfaces (Figure 4.1). Indeed, FC surfaces from line C were highly populated by *Pseudomonas* and *Curvibacter*, while NFC surfaces showed a higher abundance of *Rothia* and *Acinetobacter*. Similarly, FC surfaces from line M showed a high abundance of *Curvibacter* and *Pseudomonas* (Figure 4.1), but also *Bacillus* occurred in several samples with a relative abundance of about 1.65%

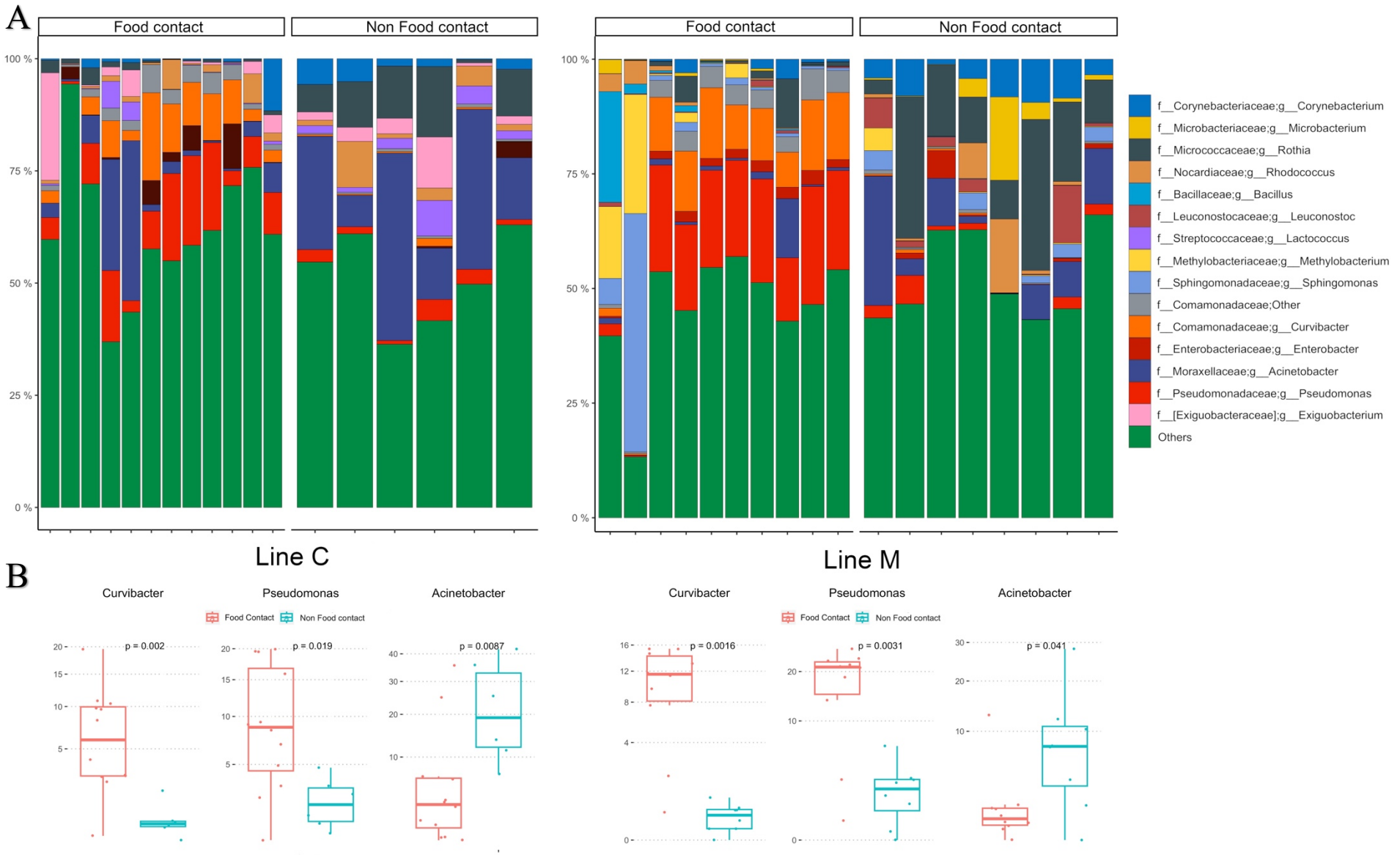

Figure 4.1 A) Bar plots showing the species with a mean relative abundance ≥ 1%. B) Boxplots showing, for each line, species with a significative different abundance between FC and NFC surfaces.

The different communities inhabiting FC and NFC surfaces were also highlighted by a principal coordinate analysis based on the Bray-Curtis distance (Figure 4.2). It was not possible to discriminate the surfaces according to the processing line (ADONIS/ANOSIM > 0.05). In addition, the Shannon's and Simpson's alpha diversity indices were not significantly different between FC and NFC surfaces in both lines (data not shown).

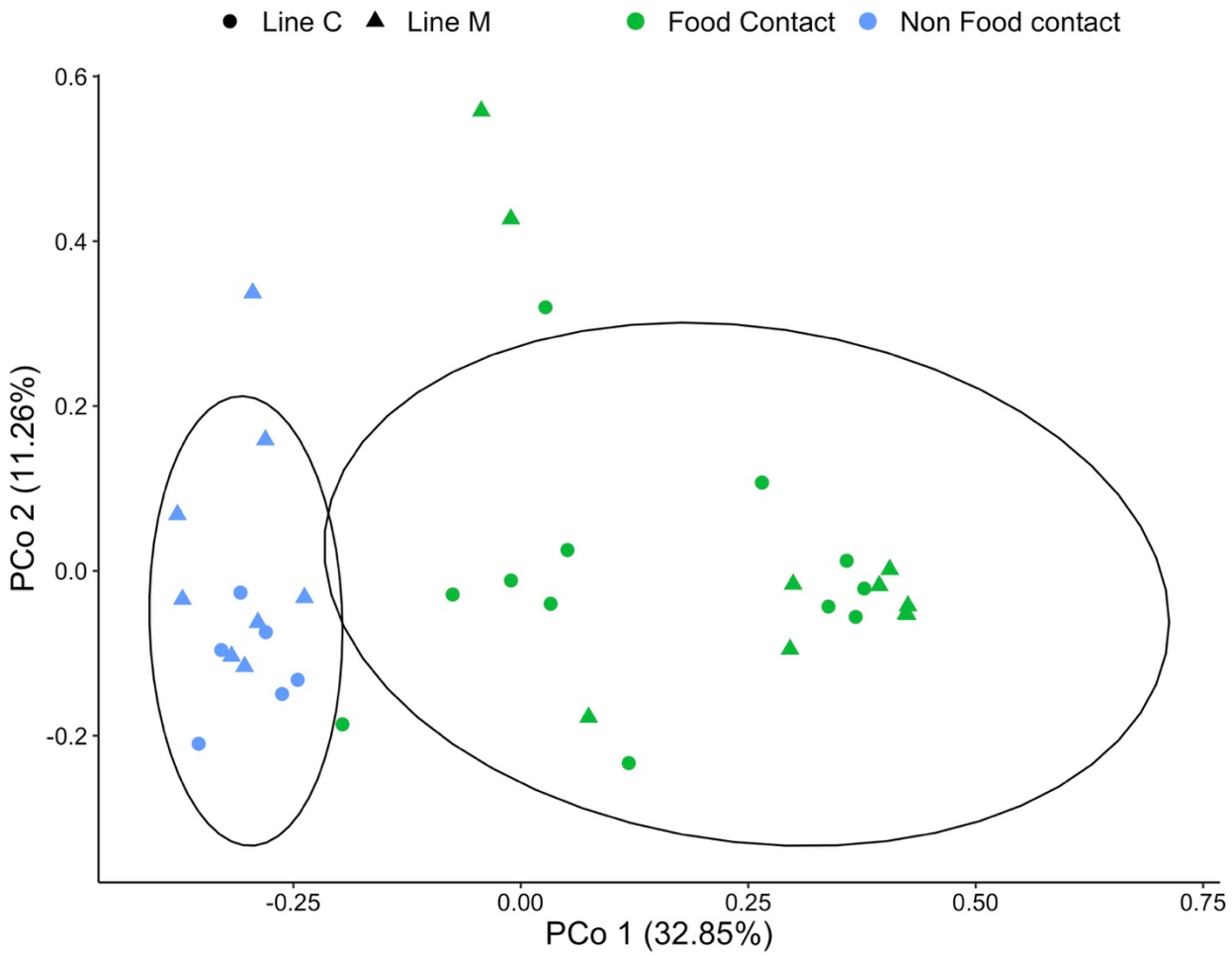

Figure 4.2 Principal Coordinate Analysis based on the Bray-Curtis dissimilarity. Samples are color-coded according to the type of surface. Shapes represent the production lines.

4.3.2. Analysis of virulence genes

Overall, we found 386 genes associated with virulence factors across 282 contigs. In addition, we observed that 111 out of 282 contigs were included in high quality MAGs, with 23 of these being included in 2 MAGs attributed to *Pseudomonas stutzeri* reconstructed from 2 surfaces from line C (Table 4.1).

Table 4.1 For each *P. stutzeri* genome, the number of genes for each virulence class is reported.

Virulence class	N. of genes	Genome	Taxonomy	Sample
Adherence	3	C3.bin.21.fa	*Pseudomonas stutzeri*	C3 - FCS
Biofilm	4	C3.bin.21.fa	*Pseudomonas stutzeri*	C3 - FCS
Effector delivery system	1	C3.bin.21.fa	*Pseudomonas stutzeri*	C3 - FCS
Motility	4	C3.bin.21.fa	*Pseudomonas stutzeri*	C3 - FCS
Adherence	3	C6.bin.12.fa	*Pseudomonas stutzeri*	C6 - FCS
Biofilm	4	C6.bin.12.fa	*Pseudomonas stutzeri*	C6 - FCS
Effector delivery system	1	C6.bin.12.fa	*Pseudomonas stutzeri*	C6 - FCS
Immune modulation	1	C6.bin.12.fa	*Pseudomonas stutzeri*	C6 - FCS
Motility	10	C6.bin.12.fa	*Pseudomonas stutzeri*	C6 - FCS

More specifically, both the MAGs showed genes linked with biofilm formation, namely *algA, algB, algC* and *algR*, as well as genes related to pilus synthesis and regulation, e.g., *pilG*, *pilJ* and *pilR*.

Interestingly, 83 out of 111 contigs were included in high quality MAGs from unknown lineages. In addition, we computed the RPKM abundance of all the virulence associated genes, and we observed that Biofilm and Motility classes were more abundant on FC surfaces than on NFC (Figure 4.3).

4.3.2. Analysis of genes encoding antibiotic resistance

208 genes encoding for genes associated with antibiotic resistance were detected in the samples. Overall, genes encoding for resistance to multiple antibiotic classes (such as fluoroquinolones, aminoglycosides and cephalosporin) were the most detected (n = 107), followed by beta-lactam (n = 28) and aminoglycoside (n = 11) resistance genes. Interestingly, 20 and 25 of these genes were linked to *Pseudomonas* and *Acinetobacter* spp., respectively. In addition, the latter was the most frequently detected genus.

Overall, the AR genes showed a greater RPKM abundance on FC surfaces than on NFC. However, by splitting the genes according to their class, genes encoding for resistance to multiple drugs were those driving the separation, being more abundant on FCS (Figure 4.4). Also, no differences emerged between the two production lines.

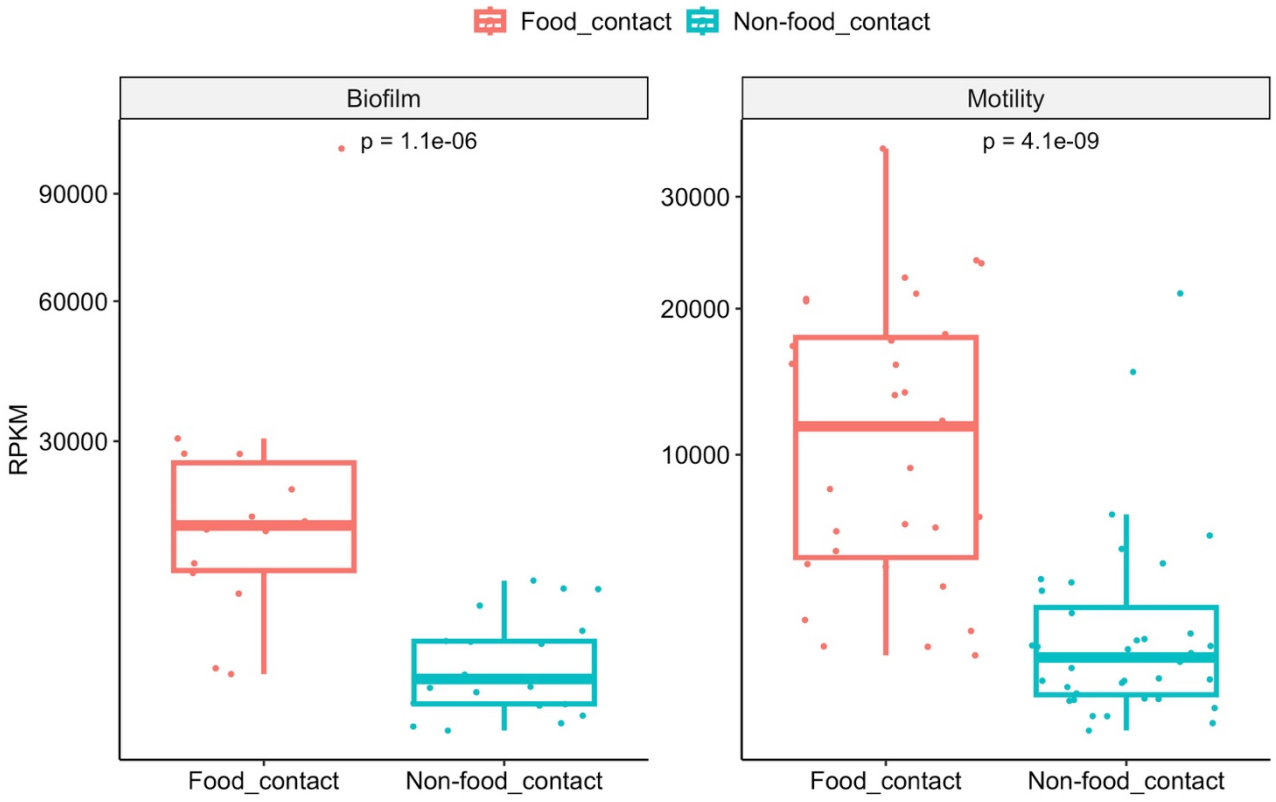

Figure 4.3 Boxplots comparing the RPKM value of the virulence genes belonging to classes "Biofilm" and "Motility" between FC and NFC surfaces.

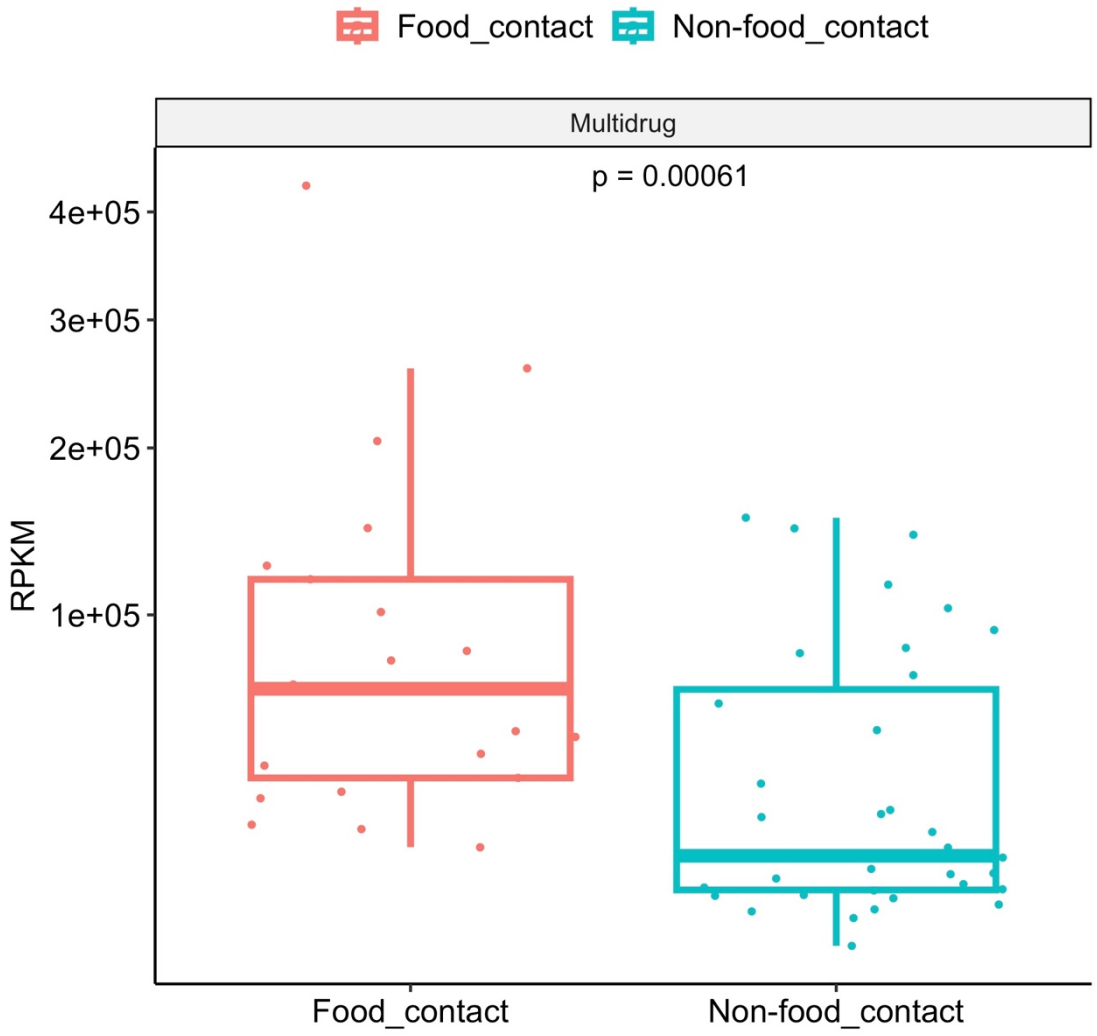

Figure 4.4 Boxplots comparing the RPKM value of the genes encoding for resistance to multiple antibiotics between FC and NFC surfaces.

4.4. Discussion

According to taxonomic analysis, very complex communities, mostly dominated by *Curvibacter*, *Pseudomonas* and *Acinetobacter*, populated the ice cream processing surfaces. *Curvibacter* spp., a Gram-negative and aerobic bacterium belonging to the family of *Comamonadaceae* (class Betaproteobacteria), have been often linked to surfaces (Gulliver et al., 2019), even though their role in these communities is mostly unknown. On the other hand, *Pseudomonas* and *Acinetobacter* have been often addressed as spoilage bacteria.

Several *Pseudomonas* species have been linked with spoilage of milk and dairy products including ice creams (Atia et al., 2022), especially due to their proteolytic activity. Indeed, Meng and colleagues (2017) estimated that ~75% of *Pseudomonas* spp. strains isolates from milk samples exhibited extracellular proteolytic activity. Also, the ability of this genus to grow and produce more biofilm at low temperatures (Kim et al., 2020) and the natural pattern of antibiotic resistance that it hosts (Camiade et al., 2020) make it very insidious for the food industry.

Similarly, some species of *Acinetobacter* spp. can grow at low temperatures (Kämpfer, 1999). Although ubiquitous, these microorganisms have been frequently associated with dairy products, and they have also been addressed as a major spoiler (Saad et al., 2018). In addition, strains showing resistance to several antimicrobial compounds widely used in humans such as penicillins (ampicillin) and cephalosporins (cefotaxime and cefepime) were isolated from bulk tank milk (Gurung et al., 2013), thus highlighting the relevance of this species for the dairy industry. Our results show that *Acinetobacter* are more abundant on NFC surfaces, although they reach a great abundance also on FC surfaces (Figure 4.1).

Consistently, we found > 45 genes attributed to *Pseudomonas* spp and *Acinetobacter* spp., which were linked to resistance to multiple biocides. Also, these genes classes were overall more abundant on FC surfaces than on NFC, thus suggesting that a selective pressure might increase the number of copies as well as their abundance (Figure 4.4).

The adaption of *Pseudomonas* spp. on food contact surfaces is also suggested by the analysis of virulence-associated genes. Indeed, several contigs showing multiple genes related to bacterial motility and biofilm formation were found on FC surfaces (Figure 4.3). In addition, we reconstructed two high-quality *Pseudomonas* sp. genomes from food contact surfaces harboring several genes associated with biofilm production and motility. Genes involved in alginate production (*algA* and *algC*) and their regulation (*algG* and *algR*; Ahmed & Ahmed, 2007) were detected in both genomes, suggesting their potential direct implication in biofilm production. Moreover, these genomes also had genes *pilG*, *pilJ* and *pilR*, which are involved in pilus synthesis and regulation (Leong et al., 2017). Although these genes are formally involved in motility of *Pseudomonas* spp., evidence suggests that their products might also be implicated in biofilm production (Leighton et al., 2018; Qing & Luyan, 2013).

Besides *Pseudomonas*, other taxa might be implicated in adherence to abiotic surfaces and biofilm formation. We observed that 83 virulence-coding contigs were included in genomes from unknown species and lineages. A similar result was also reported by Wang and colleagues (2019). Therefore, the knowledge about selection of microbial species in food industries is still limited, albeit whole metagenomic sequencing approaches might help to depict the complexity of the communities and to characterize non-culturable novel species.

Unfortunately, we lack information about gene composition of ingredients and products. However, although we are not able to detect routes of transmission of AR genes from surfaces to foods, it has been reported that surfaces might contaminate ingredients and products with potentially hazardous species. In addition, repeating the microbiome mapping routinely might help to better understand how stable these communities are and to depict temporal variations in the antibiotic resistance and biofilm formation potentials. However, to the best of our knowledge, this is the first study providing evidence of AR and biofilm formation potential in ice-creams producing manufactures. These results can be relevant for food business operators who may evaluate the

possibility to develop novel sanitation strategies to target antibiotic resistant and biofilm-forming microbial species currently overcoming cleaning and disinfection procedures.

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CHAPTER 5

Large-scale metagenomic analysis of cheeses and their production environment depicts strain-level diversity and protective genes on surfaces

5.1. Introduction

Cleaning and disinfection of surfaces are usually carried out regularly in a food processing environment (Møretrø & Langsrud, 2017). The purpose of such procedures is not only to remove food residuals, which might attract parasites, but also to reduce the number of microbes. Evidence suggests that food contact surfaces sampled after sanitation might have a contamination level of about 2.5 cfu/cm^2, which is considered an acceptable level according to suggested standards (Griffith, 2005). However, most of the strategies that have been used in the past years to assess the efficacy of sanitation procedures mostly rely on cultural methods (Aryal & Muriana, 2019), which are unable to target the unculturable fraction of the microbial community (De Filippis et al., 2021). Indeed, microorganisms might bind to food manufacture's surfaces by producing biofilms, which are made of extracellular polymeric substances (EPS; Carrascosa et al., 2021; Lorenzo et al., 2019). Notably, bacteria embedded within biofilms are more resistant to antimicrobials (including disinfectants) than free cells (Zhao et al., 2017; Xu et al., 2020; Yap et al., 2022). In addition, unculturable bacteria show a high prevalence in multispecies biofilms (Fan et al., 2020).

Presence of biofilms and selection of disinfectant-resistant microorganisms might pose a major concern for the food industry. Indeed, biofilms might host some pathogenic species, which might in turn contaminate the food product and cause foodborne diseases (Galié et al., 2018).

For example, *Listeria monocytogenes* (*Lm*) is often reported as EPS producer or as a key player in multispecies biofilms in cheesemaking facilities (Melo et al., 2015; Fagerlund et al., 2021), making food contact surfaces a reservoir of this pathogen. The persistence of *Lm* on surfaces might lead to cross-contamination, and several listeriosis outbreaks have been linked to consumption of cross-contaminated cheese (Sauders & D'Amico, 2016; Amato et al., 2017). In addition, survival or

cooperative cell-cell interactions of *Lm* in biofilms might explain its severe tolerance to antimicrobials and sanitizers (Mazaheri et al., 2021; Bland et al., 2022). However, additional pathogens have been previously linked to the dairy environment, such as *Bacillus cereus* and *Salmonella* spp. (Kousta et al., 2010; Martínez et al., 2020).

Despite these hazardous taxa, some gems might hide in microbial communities after cleaning and disinfection of dairy industrial surfaces. In fact, both starter and non-starter LAB might be autochthonous of the raw milk or originate from the food processing environment (Turri et al., 2021; McSweeney, 2007). For example, several authors hypothesized that a facility-specific residential microbiota might develop in cheese manufactures, with a potential outcome on product's quality (Quijada et al., 2018; Bokulich and Mills, 2013; Montel et al., 2014). Indeed, the cheese microbiome has a pivotal role in developing its sensorial profile, and specific flavor profiles in cheeses have been linked to selected strains (Coelho et al., 2022). In addition, although bacteriocin-producing LAB have not yet been isolated from the food processing environment, some advances in the potential use of biofilm from LAB to control spoilage and pathogenic species have been achieved (Gómez et al., 2016).

However, most of the studies describing the resistant microbial communities and its potential impact on the sensorial profile of cheeses rely on metabarcoding, i.e., the high-throughput sequencing of amplicons from taxonomically relevant genes (Liu et al., 2020). Although useful for community description, such approach is not able to reach species- and strain-level (Poretsky et al., 2014). A deeper taxonomic and functional characterization of microorganisms is needed in order to better estimate the outcomes of a specific microbial community on the cheeses characteristics, since it has been shown that different strains from the same species might have a profound impact on the product's quality (Stefanovic et al., 2017).

Therefore, the purpose of this work was to describe the taxonomic and metabolic potential of the metagenomes residing in the production environment of different types of European cheeses, as well as in ingredients and products, by performing whole metagenome sequencing. Overall, our

results show that different kind of cheeses harbor distinct microbial communities, and that different facilities producing the same cheese type host different LAB strains, which might be relevant for the "cheese fingerprinting".

5.2. Materials and methods

5.2.1. Samples collection, DNA extraction and whole metagenome sequencing

1213 samples were collected from 73 facilities across 4 European countries, i.e., Italy, Spain, Austria and Ireland. Details about the samples are reported in Table 5.1. All the facilities were visited after the routine cleaning and disinfection procedures, and before the next production shift. Environmental samples were collected by swabbing with 5 Whirl-Pak Hydrated PolyProbe swabs (Whirl-Pak, Madison, Wisconsin, US) on each surface, covering a surface of about 1 m^2 or a sampling unit (one knife, one cutting board, etc.). At the end of the production shift, ingredients (milk, whey and brine, when available) and two cheeses were collected. In addition, some of the ripened cheeses were collected at different ripening stages.

Table 5.1 Summary of the samples collected.

Country	**Surfaces**		**N. of cheeses**	**Ingredients**			**N. of facilities**
	FC	NFC		Whey	Brine	Milk	
Italy	57	25	88	16	14	16	16
Ireland	70	41	109	14	13	13	15
Austria	23	14	50	5	4	5	6
Spain	158	119	277	36	13	33	36

All the samples were pre-processed within 24 hours. Raw milk and whey samples were centrifuged at 14,000 x g for 15 minutes, and after discard of supernatant, the cellular pellet was stored at -20 °C prior further processing. Highly saline brine samples were filtered under sterile conditions using 0.2 μm membranes, which were stored at -20 °C.

Under sterile conditions, cheeses were cut into two parts, then 10 grams from the core and rind were collected into sterile bags. Each sample was diluted in 1:10 PBS 1X, homogenized in a Stomacher, then the supernatant was collected in 50 mL centrifuge tubes and centrifuged at 6500 x g for 15 minutes. The cell pellet was then stored at -20 °C prior further processing.

DNA was extracted from all the samples using the PowerSoil Pro Kit (QIAGEN, Hilden, Germany), adopting a modified version of the standard protocol previously validated to increase the total microbial DNA yield from food processing environments (Barcenilla et al., under review). The Qubit High Sensitivity kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to assess the DNA concentration of the samples, whereas whole metagenome sequencing was performed with an Illumina NovaSeq workstation (Illumina, San Diego, California, United States).

5.2.2. Bioinformatic and statistical analysis

Raw reads were quality-checked by PRINSEQ lite (version 0.20.4; Schmieder & Edwards, 2011) using parameters "-trim_qual_right 5" and "-min_len 60". High quality reads were assembled into contigs through MegaHIT (version 1.2.2, with default options; Li et al., 2016), and contigs shorter than 1500 bp were discarded. The filtered contigs were binned, and quality of bins was checked using CheckM (version 1.0.13; Parks et al., 2015). Only medium/high quality bins, i.e., those with %completeness > 90% and %contamination < 10% (Pasolli et al., 2019) were retained for further analysis. High quality bins were further clustered at 5% of dissimilarity into Species-level Genome Bins (SGBs; Pasolli et al., 2019) using MASH (version 2.0; Ondov et al., 2016). The latter tool was also used to compare the most complete and less contaminated genome from each SGB with annotated genomes from a public database, then the taxonomy of the best-matching genome was used to assign taxonomy to each SGB. FastANI (Jain et al., 2018) was used to compute pairwise distances between the genomes within each SGB, then a Principal Coordinate Analysis was performed on distance matrices.

Phylogenetic trees were produced for each SGB through PhyloPhlAn3 (version 3.0.3; Asnicar et al., 2020), using options "--accurate" and "--diversity low", and by setting species-specific databases. The refined version of the tree was then plotted using iTol (version 6.5.3; Letunic & Bork, 2021).

In addition, contigs were processed with BAGEL4 (van Heel et al., 2018), in order to find genes associated with bacteriocins production.

Taxonomic profiles of metagenomes were obtained through MetaPhlAn (version 3; Beghini et al., 2021).

5.3. Results

5.3.1. Taxonomic composition of the whole dataset

Overall, 4424 species were found across 1213 samples, with a different relative abundance. However, some representative of LAB species, i.e., *Lactococcus lactis*, *Streptococcus thermophilus* and *Lactococcus cremoris*, showed the highest mean relative abundance, with 17.88 %, 16.94 % and 11.74 %, respectively. Also, *Staphylococcus equorum*, *Brevibacterium auranticum* and *Acinetobacter johnsonii* showed a high mean relative abundance, ranging between 1.72 % and 3.77 %.

In addition, surfaces (both Food Contact and Non-Food Contact) host very diverse microbiomes, according to a PCoA based on the Bray-Curtis distance (ADONIS/ANOSIM p-values < 0.001; Figure 5.1). These differences might be mainly explained by *Kocuria* spp. and *Acinetobacter johnsonii*, which were more abundant on surfaces than in foods, as well as by *Streptococcus thermophilus* and *Lactococcus* spp., that dominated ingredients and products (Figure 5.1). In addition, Shannon and Simpson's alpha diversity indices were significatively higher in surfaces

samples than in ingredients and products, suggesting that the food manufacture environment might be home of a wider plethora of species (Figure 5.2).

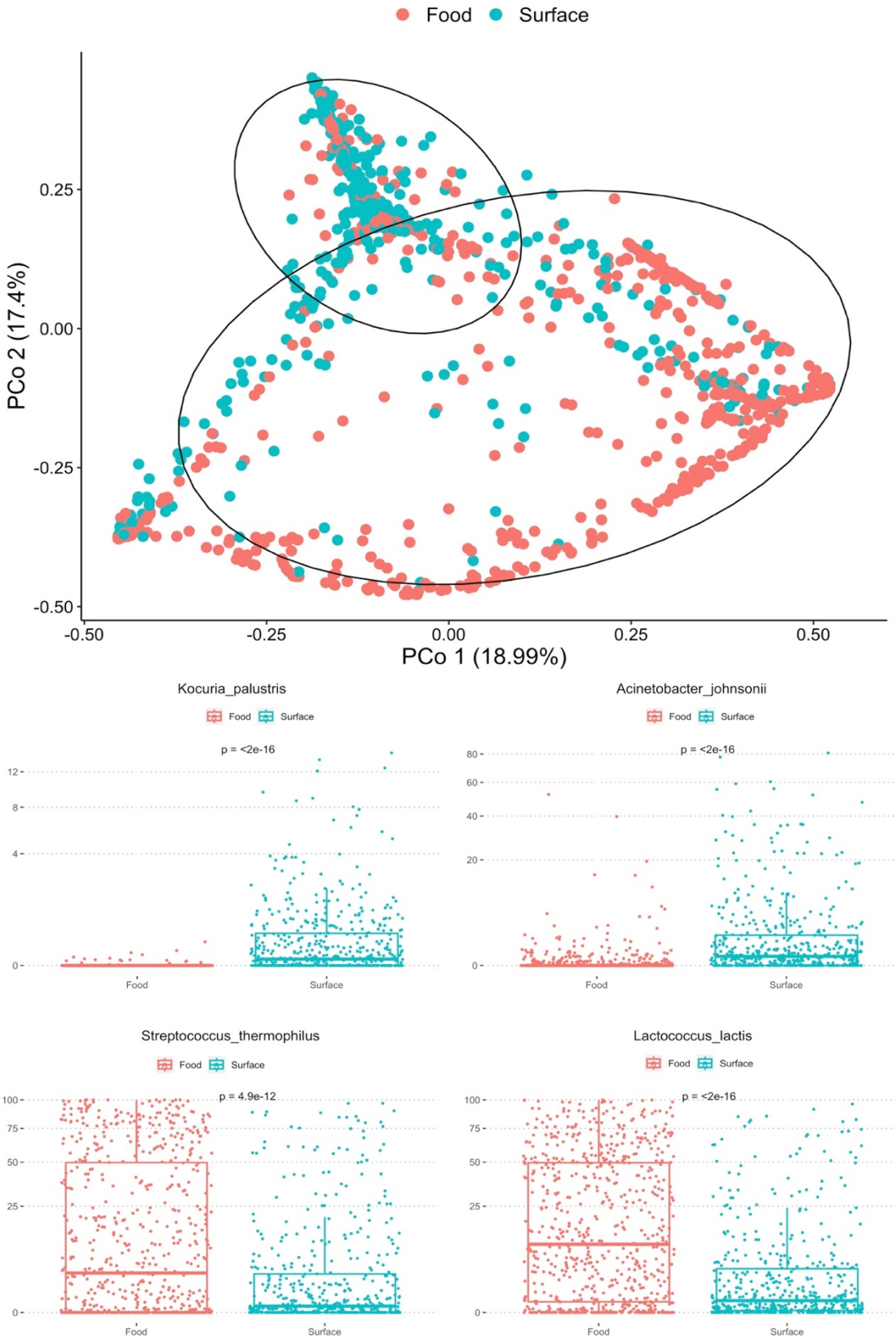

Figure 5.1 PCoA and boxplots highlighting taxonomic differences between surfaces (FC and NFC) and foods.

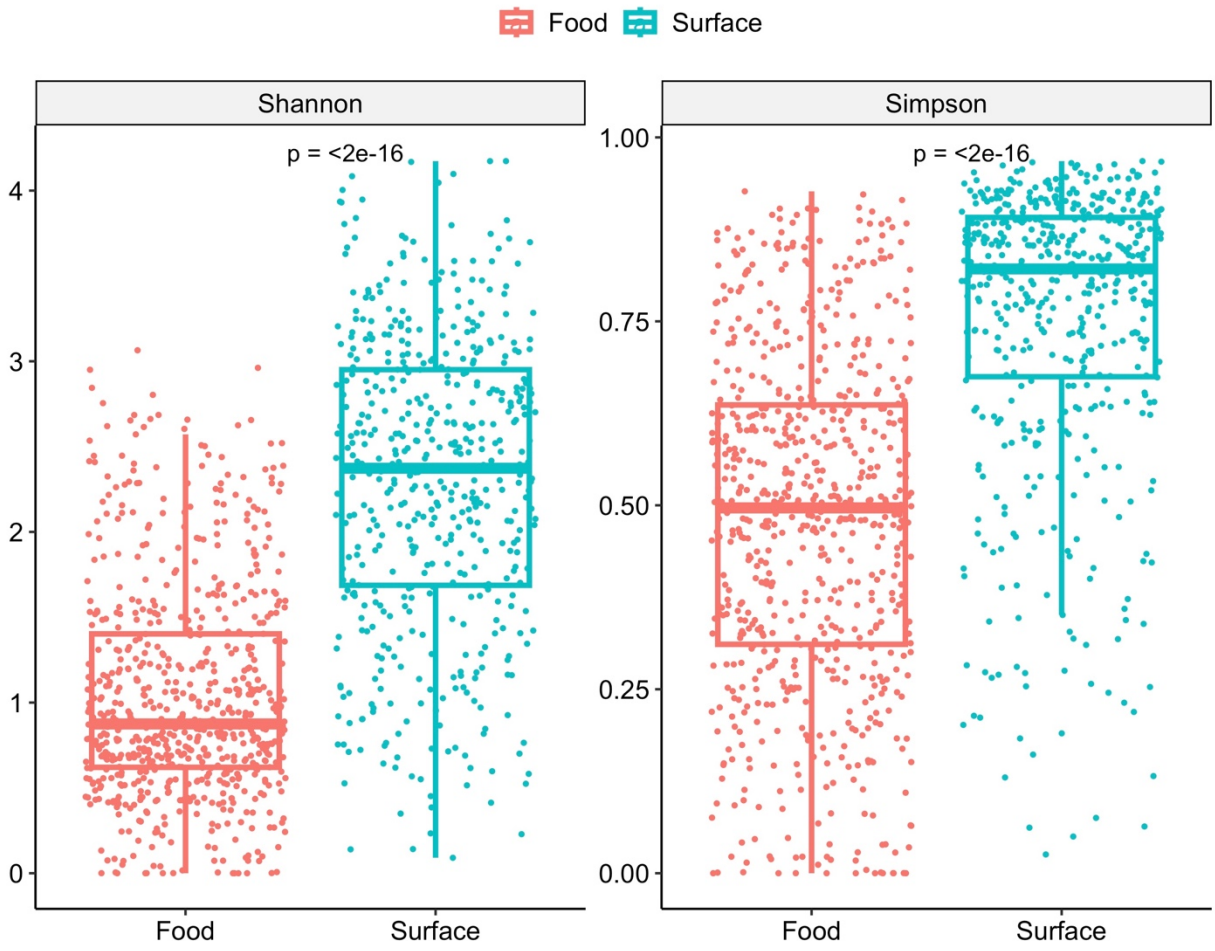

Figure 5.2 Boxplots comparing the alpha-diversity indices between foods and surfaces.

5.3.2. Taxonomic composition of surfaces

We also decided to further explore the taxonomic composition of surfaces. Also in this case, PERMANOVA and Analysis of Similarities highlighted a statistically significant difference between FC and NFC surfaces, as well as between production areas (e.g., processing room, packing room, etc.; $p < 0.001$), although no clear clustering of samples emerged from the PCoA (data not shown).

Acinetobacter johnsonii, *Kocuria carniphila*, *Kocuria palustris, Kocuria salsicia* and *Paracoccus haeundaensis* were present with a relative abundance > 0.01 % in at least 70% of NFC from processing area samples, whereas the most prevalent species on molding machines, cheese vats

and curd shredders/draining tables were LAB, namely *Lc. lactis*, *Lc. cremoris* and *S. thermophilus*. However, also on FC surfaces *Acinetobacter johnsonii* was highly prevalent (Figure 5.3A). On the contrary, *Brachybacterium alimentarium*, *B. tyrofermentans* and *Brevibacterium auranticum* were more prevalent on FC/NFC surfaces from the ripening area, together with *Staphylococcus equorum* (Figure 5.3B).

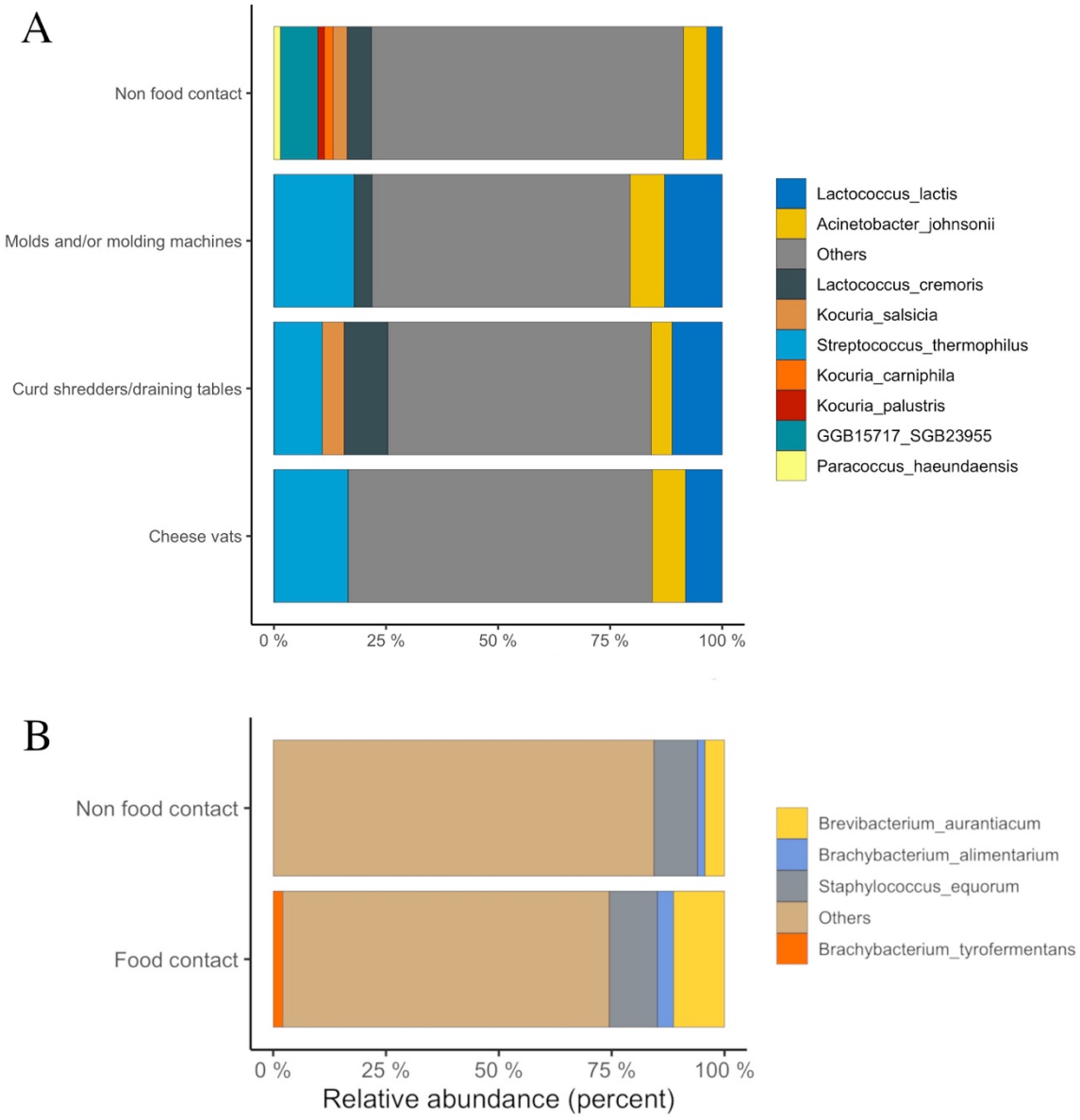

Figure 5.3 Barplot showing the species with abundance > 0.1% in at least 50% of each group's samples in A) processing and B) ripening areas.

5.3.3. Taxonomic composition of foods

According to a hierarchical clustering performed on raw milk, whey and brine taxonomic profiles based on the "Correlation" metrics, raw milk samples cluster separately, regardless of the host (Figure 5.4). *Streptococcus thermophilus* is among the species driving the separation, since it has

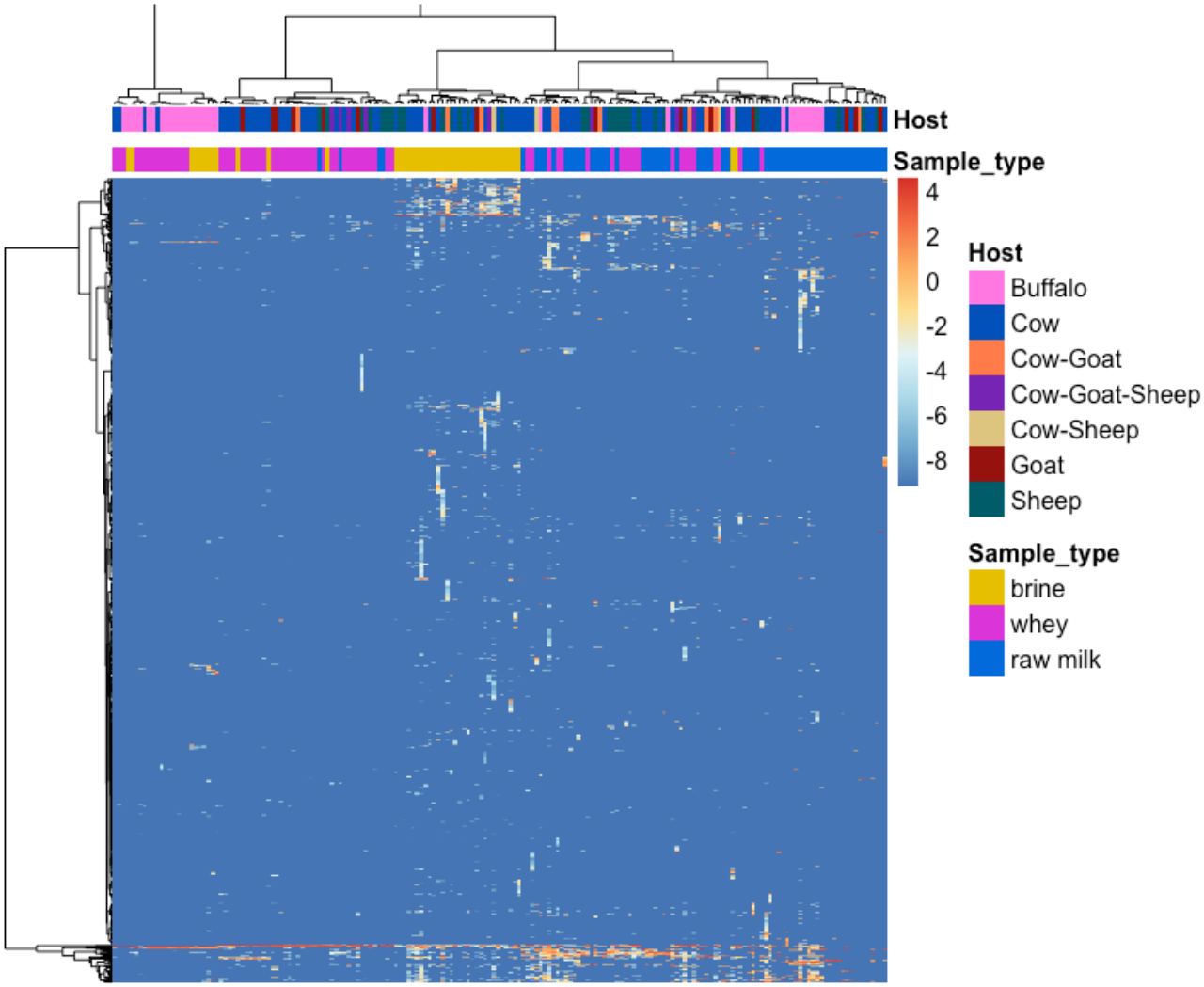

Figure 5.4 Hierarchical clustering of taxonomic profiles of ingredients based on the "Correlation" metric.

a very low abundance in raw milk samples, albeit being prevalent in brine and whey samples. In addition, *Chromohalobacter japonicus* was exclusively found in brine samples, thus representing a signature of the group.

Interestingly, principal coordinate analyses based on Bray-Curtis distance suggest that facility-specific microbial communities might develop in different manufactures producing the same

cheese type Figure 5.5. However, minor taxa are responsible for the discriminant clustering of samples from different facilities (data not shown).

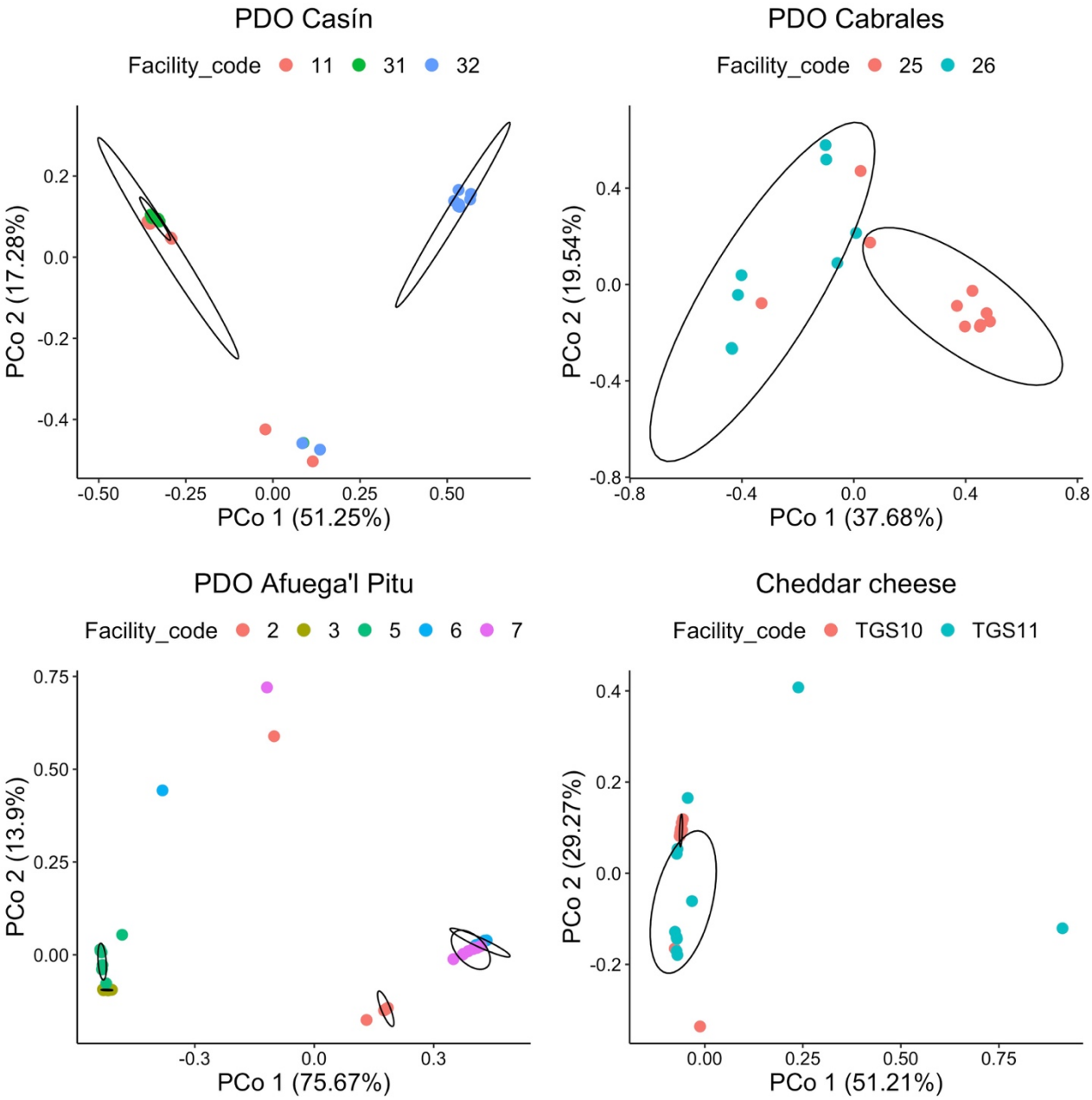

Figure 5.5 PCoAs based on Bray-Curtis distance performed between samples collected in different facilities producing the same cheese type.

5.3.4. Cheese- and facility-specific LAB strains with unique metabolic potential are selected in different industries

Table 5.2 reports the SGBs on which we focused for strain-level analysis.

Overall, 360 high quality *Streptococcus thermophilus* bins were reconstructed from foods and surfaces from Caciocavallo cheese (n = 50), Mozzarella cheese (n = 41), Gamoneu cheese (n = 34), Afuega'l Pitu cheese (n = 23) and Cheddar cheese (n = 19) facilities. Interestingly, phylogenetic analysis highlighted that genomes reconstructed from facilities producing the same cheese type mainly fall in the same clade (Figure 5.6A).

Table 5.2 SGBs interesting for strain-level analysis.

SGB	N. of MAGs	Taxonomy
SGB_12	362	*Streptococcus thermophilus*
SGB_77	138	*Leuconostoc mesenteroides*
SGB_795	97	*Leuconostoc pseudomesenteroides*
SGB_0	96	*Lactobacillus delbrueckii*
SGB_1147	82	*Lactobacillus helveticus*
SGB_228	76	*Corynebacterium casei*
SGB_575	57	*Macrococcus caseolyticus*

A similar pattern was also observed for *Lactobacillus delbrueckii*, as well as for other NSLAB, such as *Corynebacterium casei*, *Leuconostoc mesenteroides*, *Lactobacillus helveticus*.and *Macrococcus caseolyticus*.

Furthermore, for most of these species a clear clustering of the bins according to the facility emerged from both phylogenetic analysis (Figure 5.6B) and ANI distance-based PCoA.

To test whether the facility-specific strains had different metabolic potential, we performed a pangenome analysis. Therefore, for each strain we obtained a presence-absence profile of the genes from the species' pangenome. A hierarchical clustering of these profiles based on the binary distance confirmed our hypothesis for several species, such as *Lactobacillus delbrueckii* (Figure 5.7). Also, we were able to identify those genes that contributed to the clustering, and we observed that several of them were involved into proteolysis and lipolysis. Indeed, acyltransferases were

more prevalent in *Lb. delbrueckii* strains reconstructed from Mozzarella cheeses than from other types of cheeses.

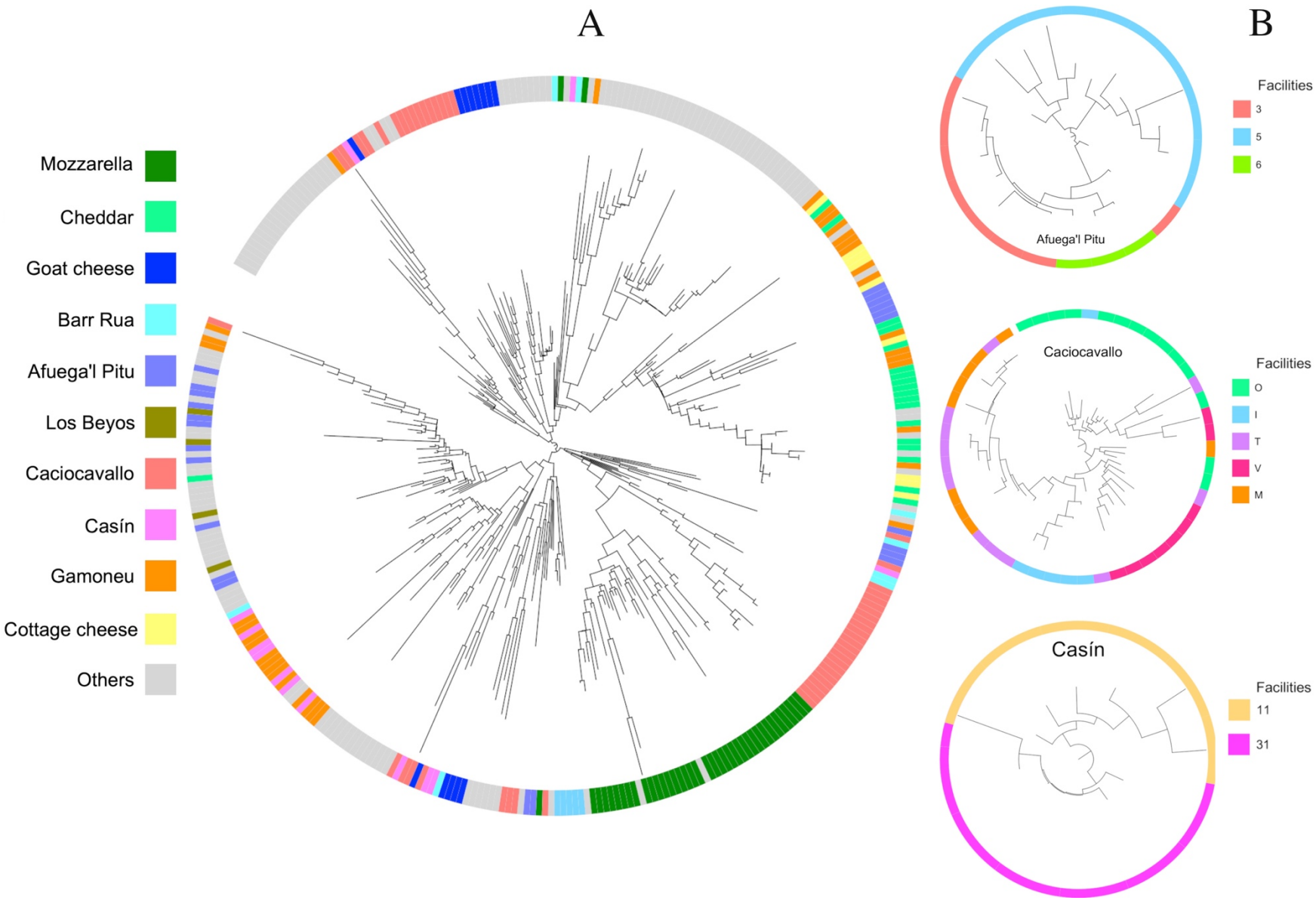

Figure 5.6 A) Phylogenetic tree of all the MAGs attributed to *Streptococcus thermophilus*. MAGs are color-coded according to the cheese type. B) Phylogenetic trees showing facility-specific *S. thermophilus* strains.

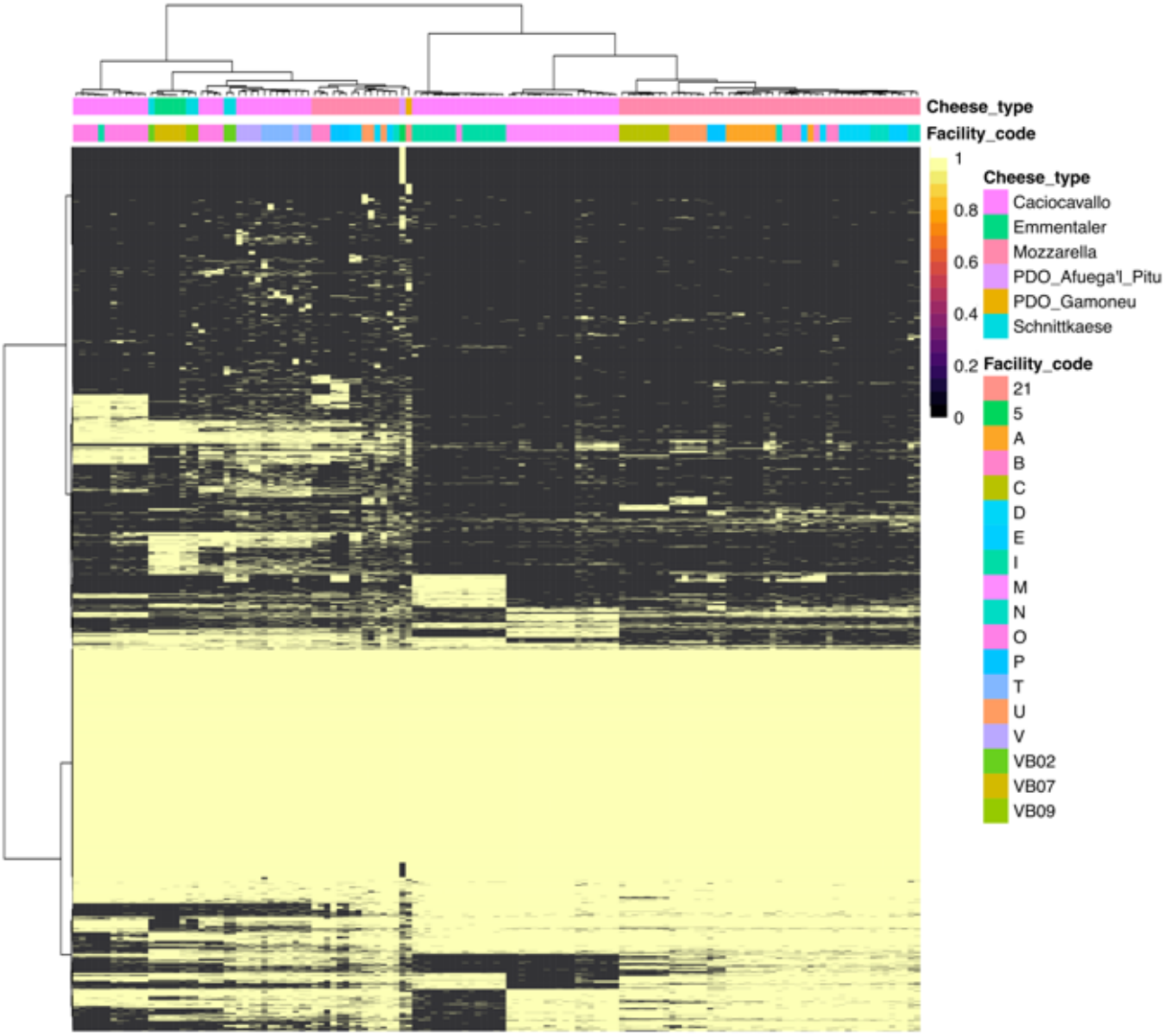

Figure 5.7 Hierarchical clustering of the *Lactobacillus delbrueckii* genomes based on the presence-absence of genes from the species' pangenome.

5.3.5. Industrial surfaces host bacteriocin-producing LAB

Overall, BAGEL4 detected 8832 genes associated with bacteriocin production in the whole dataset. Most of these genes were reconstructed from the surfaces (both FC and NFC), with 5126 calls overall, whereas 3427 genes were detected in foods (including ingredients and products). On surfaces, the most prevalent classes of bacteriocins were colicins (with 3.62 occurrences per FC and 3.02 per NFC on average), helveticins (2.70 per FC and 2.40 per NFC), linocins (2.67 per FC and 2.82 per NFC) and zoocins (2.43 per FC and 3.56 per NFC) (Figure 5.8).

In the same vein, the group of helveticins was among the most prevalent in brine and cheeses samples, with an average of 3.25 and 2.11 occurrences per sample (± 1.22 and ± 0.77, respectively), whereas colicins were frequently detected in raw milk samples (3.43 ± 2.76 genes per sample on average).

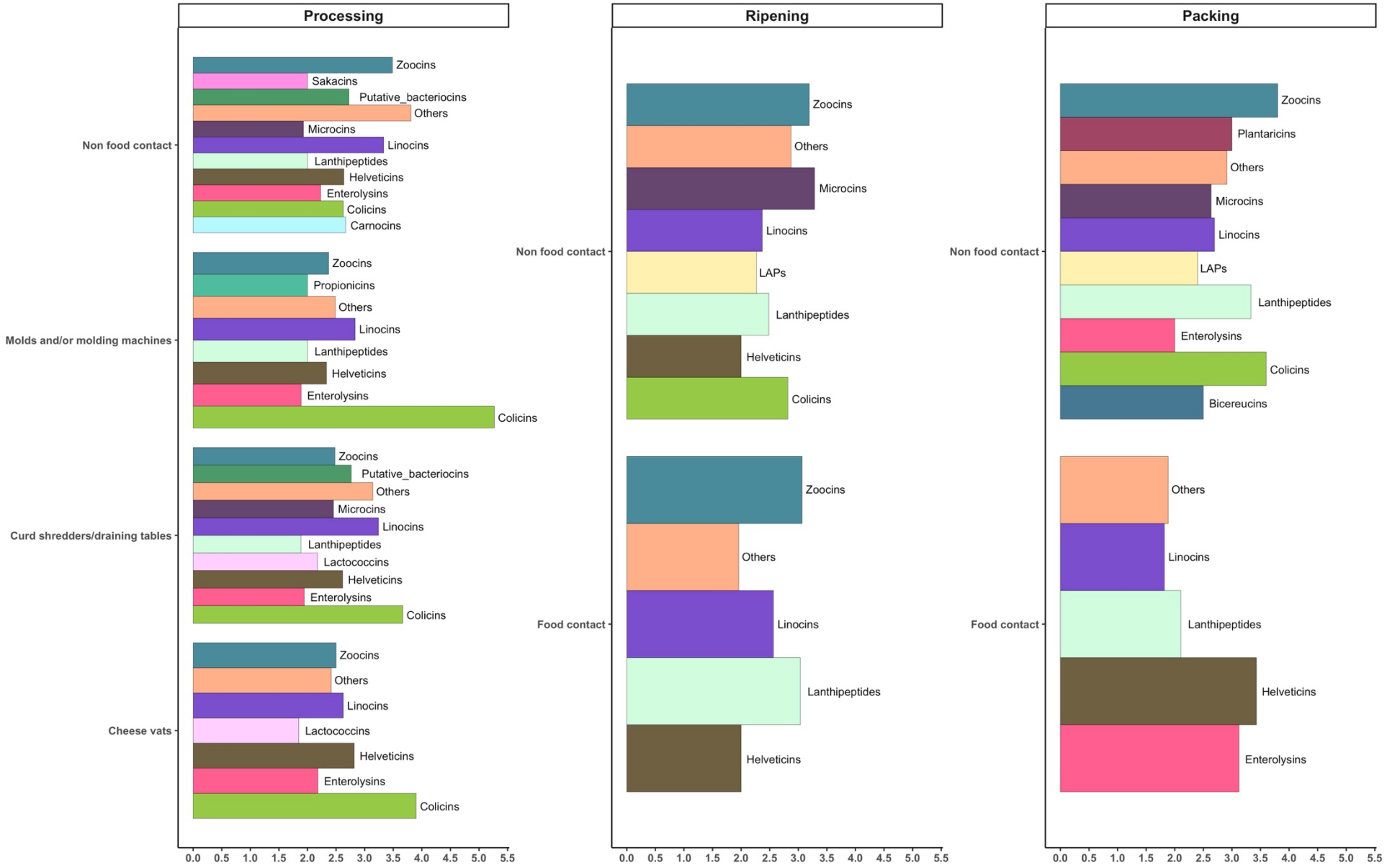

Figure 5.8 Barplot showing the average number of copies of bacteriocins-associated genes for each group of samples.

5.4. Discussion

To the best of our knowledge, this is the first large-scale study on cheeses microbiome and on their associated production environment, including products from different technological settings and countries.

Lactic Acid Bacteria have a determinant role in defining the sensory and quality characteristics of cheeses (Ercolini, 2020). Among the LAB present in cheeses, *Streptococcus thermophilus* and *Lactococcus lactis* are among the most recurrent, also showing a great relative abundance (Walsh et al., 2020). Although mainly used as starter cultures and selected for their pro-technological properties (e.g., production of exopolysaccharides, resistance to bacteriophages and to high concentration of salts; Fox et al., 2017), these species also contribute to shaping the sensorial profile of dairy products. For example, strains of *Streptococcus thermophilus* express enzymes involved in amino acid degradation, such as branched-chain aminotransferase (BcAT), phosphotransacylase (PTA) and alcohol dehydrogenase (Cui et al., 2016) and they might contribute to defining the aromatic bouquet of fermented milk and cheeses (Dan et al., 2018), whereas *Lactobacillus delbrueckii* was linked to higher concentration of esters and ketones in hard and cooked cheeses (Buchin et al., 2017). Therefore, the high abundance of these species in final products, brine and whey samples is not surprising. In addition, these species were also found on surfaces, highlighting their ability to resist to cleaning and disinfection procedures and their willingness to adhere to abiotic surfaces. It was previously observed that some LAB strains isolated from surfaces after sanitation show tolerance to Quaternary Ammonium Compounds (Sidhu et al., 2001), and Fernández Márquez and colleagues (2017) confirmed the result, showing that *Lactococcus* spp. isolated from a dairy plant show tolerance to triclosan, cetrimide and benzalkonium chloride. The repeated exposition of these microbes to sub-inhibitory sanitizers concentrations might explain the establishment of these specific taxa on the food contact surfaces that we sampled (Flores-Vargas et al., 2021).

Members of the genera *Brachybacterium* and *Brevibacterium*, which were highly prevalent on surfaces of ripening areas, are reported to contribute to the final coloration of cheeses, since they are able to produce carotenoids such as isorenieratene, 3-hydroxy-isorenieratene and 3,3′-di-hydroxy-isorenieratene (Irlinger et al., 2017), and they are particularly relevant during maturation of smear-ripened cheeses (Monieu et al., 2005), whereas *Staphylococcus equorum* has been linked with production of flavor and with antibacterial activity against *Listeria monocytogenes* (Haastrup et al., 2018).

Acinetobacter spp. have been frequently linked to food processing environment and equipment (Møretrø & Langsrud, 2017; Malta et al., 2020). Particularly, *A. johnsonii* has gained attention in recent years due to its range of antibiotic-resistance genes (especially beta-lactamases) and to the presence of several plasmids which enhance spread/acquisition of antimicrobial resistance genes and its ability to adapt to various niches (Montaña et al., 2016).

Although research mostly focuses on the negative outcomes linked to surfaces-adapted microorganisms, we observed that several LAB from the production areas harbor multiple copies of bacteriocin production genes. Indeed, colicins were the most prevalent on surfaces from the production areas, frequently occurring on molding machines and cheese vats (Figure 5.8). Colicins are bacteriocins active against *E. coli*, and they usually induce cell death by forming pores in the cell membrane (Kleanthous, 2010). Interestingly, Rendueles and colleagues (2014) described a colicin that selectively targets microorganisms within biofilms, thus potentially reducing the EPS layer and inhibiting the adherence of pathogens. However, also helveticins and linocins were frequently detected on FC surfaces. In particular, linocins are produced by *Brevibacterium* spp., and they inhibit the growth of *Listeria* spp. and other coryneforms (Valdés-Stauber & Scherer, 1994). The presence of bacteriocin-producing LAB species on surfaces might represent an advantage for food business operators. Indeed, it has been shown that biofilm made of potential probiotic species (namely LAB), are capable of reducing the establishment of pathogens on abiotic

surfaces (Gómez et al., 2016; Stellato et al., 2015), and the fine-tuning of detergents containing protective LAB has been hypothesized (Falagas & Makris, 2009).

Recently, metagenomic-based strain-level analysis has depicted the diversity occurring in several areas (New & Brito, 2020), and it has also been used to describe the strains co-existing in cheeses (Yang et al., 2021). Our results support this idea and show the potential of whole metagenomic sequencing to discriminate putative LAB subclades. Indeed, our results suggest that some SLAB and NSLAB subclades are strongly linked to different cheese types (Figure 5.6A). This confirms previous evidence from Andrighetto et al. (2002), which highlighted a wide heterogeneity between *S. thermophilus* strains from different Italian Protected Designation of Origin (PDO) cheeses, and it has been hypothesized that strain-level characterization of some LAB species might be useful to discriminate between PDO cheeses (Cardin et al., 2022).

In addition, although genotypic diversity between strains does not always correspond to functional diversity, we observed a very clear functional differentiation between strains reconstructed from different types of cheeses, according to pangenome analyses (Figure 5.7). This result supports the idea that subclades of some LAB species might exert different activities in different cheese types. Also, some of the genes discriminating between the subclades are involved in aroma production. For example, some acyltransferases, which catalyze the production of esters, occurred in all the *Lb. delbrueckii* genomes from Mozzarella cheese facilities, while lacking in the others. However, these results should be further integrated with cheeses proteomics and metabolomics, to better explore correlations between strains and substrates/metabolites.

Moreover, we noticed that, within a cheese type, genotypic and phenotypic profiles of LAB strains from the same facility clustered together, and separately from the others (Figure 5.6B), which was also observed at functional level (Figure 5.7). Therefore, the selection of facility-specific microbial strains from multiple LAB species might be linked with unique sensorial profiles potentially discriminating between the same cheeses from different industries, since production of aroma compounds and textures are strain-specific more than species-specific, and also depend on their

interactions (McAuliffe et al., 2019). Bokulich and colleagues (2013) reached the same conclusion, although their study was based only on the metataxonomic characterization of the communities. Also, it is likely that the environmental conditions and stresses specific to each industry lead to selection of well-established LAB strains with a diverse potential.

In addition, the detection of facility-specific strains might be linked to the discriminating taxonomic profiles across industries producing the same cheese type (Figure 5.5). Indeed, there's increasing evidence of the perturbance effects of a single strain on species-level composition of microbial communities (Niccum et al., 2020).

Taken collectively, these results suggest that despite the resistance of potentially hazardous microorganisms on surfaces and tools, communities dominated by bacteriocin-producing LAB might exert a protective effect, inhibiting the adherence and the establishment of pathogens. In addition, our results provide evidence of environmentally selected facility-specific LAB strains with a diverse metabolic potential, which might represent a valuable tool for cheese fingerprinting.

5.5. References

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CHAPTER 6

Patterns of antibiotic resistance and mobilization in the food industry

6.1. Introduction

One of the most important prerogatives of microorganisms is to adapt to the environment in which they live (Gao et al., 2011). Environmental stresses such as variation in pH, temperature or in concentration of compounds exert a selective pressure on bacteria that, in some cases, lead to development of new phenotypes through several mechanisms, such as horizontal gene transfer and gene recombination (Wani et al., 2022). In addition, due to their short generation times and versatile genomes, microbes might adapt to environmental stresses in a very short time (Koskella & Vos, 2015). The extensive use of antibiotics is a great example of environmental stress to which microorganisms are exposed. In fact, even though several bacterial species developed antibiotic tolerance before the world scale production of these drugs (D'Costa et al., 2011), it was with their extensive use in clinical and domestic settings that microorganisms enhanced the mobilization and amplified the range of resistance (Larsson & Flach, 2022).

Nowadays, antibiotic resistance is a very harsh problem, and 1.2 million deaths worldwide were attributed to resistant bacteria in 2019 (Murray et al., 2022). The resistant species most frequently involved in infections are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp., also designed by the World Health Organization as the ESKAPE group (Mancuso et al., 2021). These species have developed multiple mechanisms to tolerate and escape the effects of antibiotics. For example, the *A. baumannii* genome naturally codifies for the *ampC* gene which neutralizes the effect of cephalosporins beta-lactam antibiotics, but selective pressure also led to increasing the expression and lowering the specificity of efflux pumps, making the bacterium resistant to multiple class of drugs (Abdi et al., 2020).

Although frequently isolated from clinical settings, it is reported that environmental reservoirs of these species are soil, foods (especially fruit, vegetables and animals), rivers and drinking water (Denissen et al., 2022). In addition, antibiotic resistance and virulence genes are commonly plasmid-coded in the ESKAPE pathogens. Even though the vast majority of horizontal gene transfer events occur between phylogenetically related clades (Botelho et al., 2022), there is evidence of genetic material exchange involving phylogenetically distant microbes (Redondo-Salvo et al., 2020; Emamalipour et al., 2020), which might explain the emerging of new resistant species.

Antibiotic resistant microbes or genes might reach the human body through food, as it has been recently reported (Camellini et al., 2021). Contamination of products with antibiotic resistant genes or microbes might occur throughout the food chain, from pre-harvest to processing of ingredients.

For this reason, the EFSA panel on Biological Hazard recently assessed the role of the food production environment in selecting and spreading antimicrobial resistance (EFSA panel on Biological Hazard, 2021). The panel reported that contamination of food contact surfaces might carry antibiotic resistance genes, and also suggested that the use of biocides might exacerbate AR selection (EFSA panel on Biological Hazard, 2021).

Preliminary data about the distribution of antibiotic resistant taxa in food processing environments are available. For example, Wiktorczyk-Kapischke and colleagues (2021) reviewed the response mechanisms of *L. monocytogenes* to stresses exerted in food processing facilities, highlighting that resistance to disinfectants and existence in biofilms might promote absorption of plasmids containing ARGs, whereas Pamuk et al. (2022) tested the antimicrobial resistance of six *Salmonella* spp. isolated from a canteen, observing that all of them were resistant to multiple commonly used antibiotics such as penicillin/novobiocin, amoxicillin and vancomycin.

Despite these efforts, a large-scale systematic analysis of occurrence of antibiotic resistance genes and taxa in the food industry is still lacking. Therefore, the purpose of this work is to describe

pattern of the spread of antimicrobial resistance in the food industry, focusing on the most relevant taxonomic groups and on their resistance patterns.

6.2. Materials and methods

6.2.1. Samples collection, DNA extraction and whole metagenome sequencing

Overall, 1768 samples were collected across 5 European countries, i.e., Italy, Spain, Austria, Ireland and Iceland.

Our data are skewed. Most of the samples are from dairy industries (1463 samples overall), followed by fermented sausages (95 samples), cured meats (73 samples), processed fish (57 samples), raw meat (31 samples), minimally processed vegetables (29 samples) and aged beef (20 samples) processing facilities, and a total of 87 industries were visited. Samples were collected from surfaces (both FC and NFC) of different areas (e.g., processing, stocking, ripening), and from ingredients and products.

Samples from surfaces were collected at the end of cleaning and disinfection. Ingredients and products were collected from the very first production shift following the environmental sampling. Environmental samples were collected by swabbing with 5 Whirl-Pak Hydrated PolyProbe swabs (Whirl-Pak, Madison, Wisconsin, US) on each surface, covering a surface of about 1 m^2 or a sampling unit.

All the samples were transported to the laboratory at 4 °C and pre-processed within 24 hours. Liquid samples (e.g., raw milk and whey) were centrifuged at 14.000 x g for 15 minutes, and the cellular pellet was stored at -20 °C prior further processing.

Cheeses were sampled as previously described (see paragraph 5.2). About 20 g of all the other products (raw and cured meats, fermented sausages, fish and vegetables) were weighted, suspended in 1:10 Phosphate Buffered Saline (PBS) 1X and hand-massaged to enhance transfer of cells from the food to the liquid.

DNA was extracted from all the samples using the PowerSoil Pro Kit, with some minor modification to enhance the extraction yield (Barcenilla et al., under review). After assessing the

DNA concentration of each sample using the Qubit HS assay, whole metagenome sequencing was performed with an Illumina NovaSeq workstation (Illumina, San Diego, California, United States).

6.2.2. Bioinformatic and statistical analysis

Raw sequences were quality-checked using PRINSEQ lite (version 0.20.4; Schmieder & Edwards, 2011) with parameters "-trim_qual_right 5" and "-min_len 60". After filtering out low quality reads, the remaining sequences were assembled into contigs using MegaHIT (version 1.2.2; Li et al., 2016). Contigs shorter than 1.000 bp were not included in further analyses. Taxonomy of each contig was inferred with Kraken2 (Wood et al., 2019).

Contigs were then screened for antibiotic resistance genes using TORMES (version 1.3.0, Quijada et al., 2019), relying on the ResFinder (Florensa et al., 2022), CARD (Alcock et al., 2023) and ARG-ANNOT (Gupta et al., 2014) databases. Only alignments with percentage of identity and coverage >= 80% were considered. Platon (Schwengers et al., 2020) and PlasFlow (Krawczyk et al., 2018) were used to infer whether a contig contained parts of plasmids, while MGEfinder (Durrant et al., 2019) screened the contigs for integrative mobile genetic elements. Also, WAAFLE (version 0.1.0) detected whether contigs were involved in Lateral Gene Transfer events. To assess the abundance of each ARG, high quality reads were mapped against a dereplicated version of the ResFinder database using BowTie2 (version 2.2.9; Langmead & Salzberg, 2012) with options "--no-unal", "--very-sensitive" and "--end-to-end", while ViromeQC (Zolfo et al., 2019) inferred the percentage of bacterial reads from each metagenome. Therefore, Copies Per Million (CPM) of each gene were calculated normalizing the number of reads mapping to each gene for the percentage of bacterial reads in the metagenome.

Statistical analyses and plotting were performed in a R environment (https://www.r-project.org, version 4.1.3). All the plots were produced using the package 'ggplot2'. The χ^2 test was performed using the function 'chisq.test' from the 'stats' package, and the plots of residuals was plotted using the function 'corrplot' from the so-called package. Finally, the median CPM abundance of ARGs was compared between the groups through the Wilcoxon' rank sum test ('wilcox.test' R function).

6.3. Results

6.3.1. Acinetobacter and Staphylococcus spread ARGs on industrial surfaces

50% of the contigs had a length included between 2000 and 7000 bp, with a median value of 3387. According to TORMES, 704 samples out of 1768 harbored at least one antibiotic-resistance gene. Since the number of samples from cheesemaking facilities outstood the other industry types, we calculated the per-sample number of ARGs, by normalizing the number of occurrences within a specific 'Surface_type per Facility_type' combination by the amount of samples falling into that group.

In general, NFC and FC surfaces harbored the highest number of ARGs, with 11.48 and 7.57 coding sequences per sample, respectively. Among the AR classes, Aminoglycosides were the most widespread (2105 occurrences), followed by beta-lactams (1728 occurrences) and tetracyclines (especially in facilities producing meats, i.e., 'Aged beef', 'Cured meats' and 'Raw meats', where 3.44, 2.59 and 1.84 genes per sample were found, respectively). Notably, also genes coding for resistance to multiple antimicrobials were widespread, with 753 occurrences.

We also assessed the most contributing taxa (Figure 6.1). Overall, 1763 CDS were attributed to *Staphylococcus* spp, 1411 to *Acinetobacter* spp. and 492 to *Enterococcus*. Interestingly, 1373 CDS were labelled as 'Unclassified'.

Acinetobacter contributed the most to the spread of beta-lactams, with 629 occurrences overall, followed by *Aeromonas* and *Staphylococcus*. However, *Acinetobacter* spp. also carried several aminoglycosides (n = 337). Moreover, *Staphylococcus* spp. mostly carried tetracycline (n = 335) and genes responsible for multidrug resistance (n = 209). The Chi-squared test further confirmed that the taxa don't contribute in the same way to ARGs (p-value < 2.2×10^{-16}). In particular, the correlation plot based on the Pearson's correlation index (Figure 6.2) confirmed that *Acinetobacter* and *Aeromonas* are strongly correlated with beta-lactams, but also evidenced that *Brevibacterium* spp. carry amphenicols-resistance genes and that *Klebsiella* spp. are resistant to Fosfomycin.

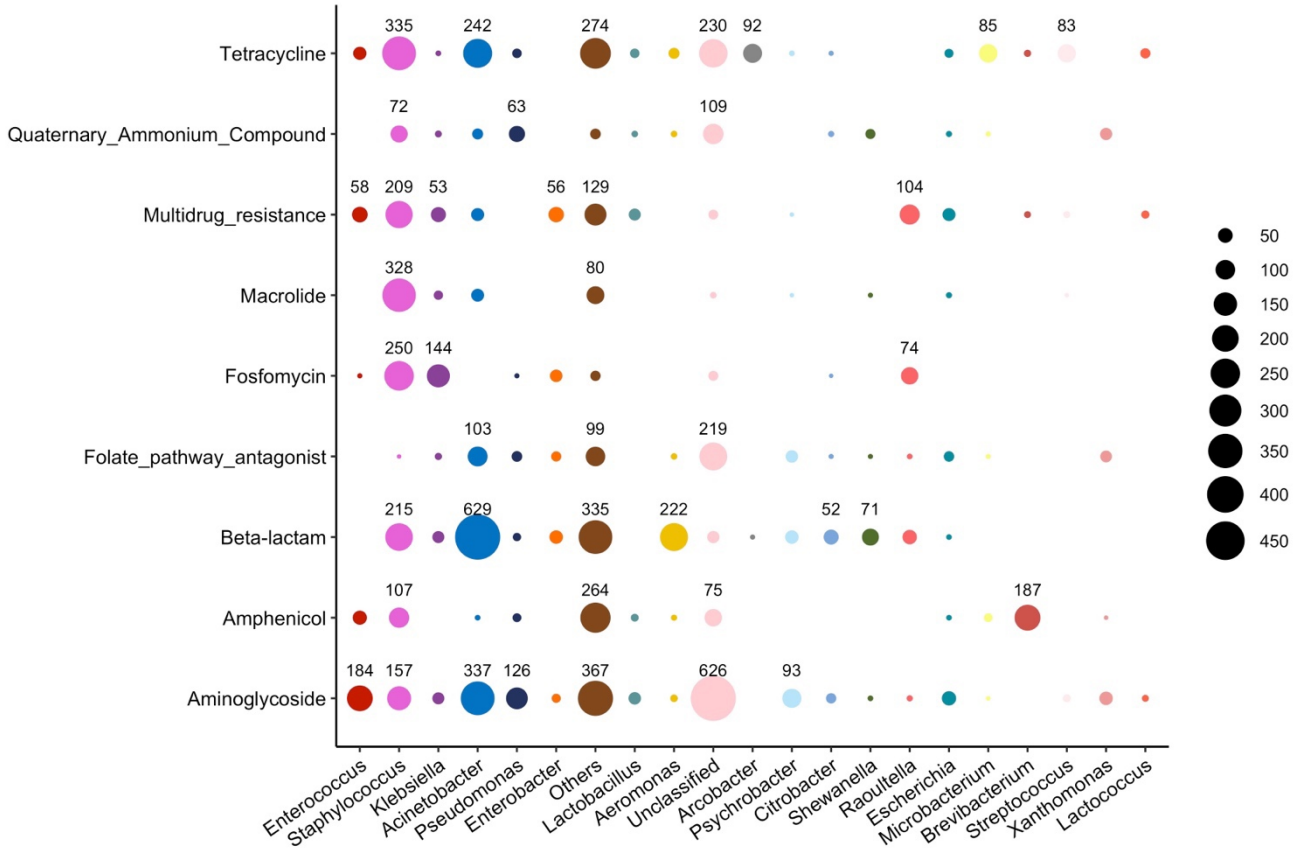

Figure 6.1 Bubble plot showing the number of occurrences attributed to each taxon.

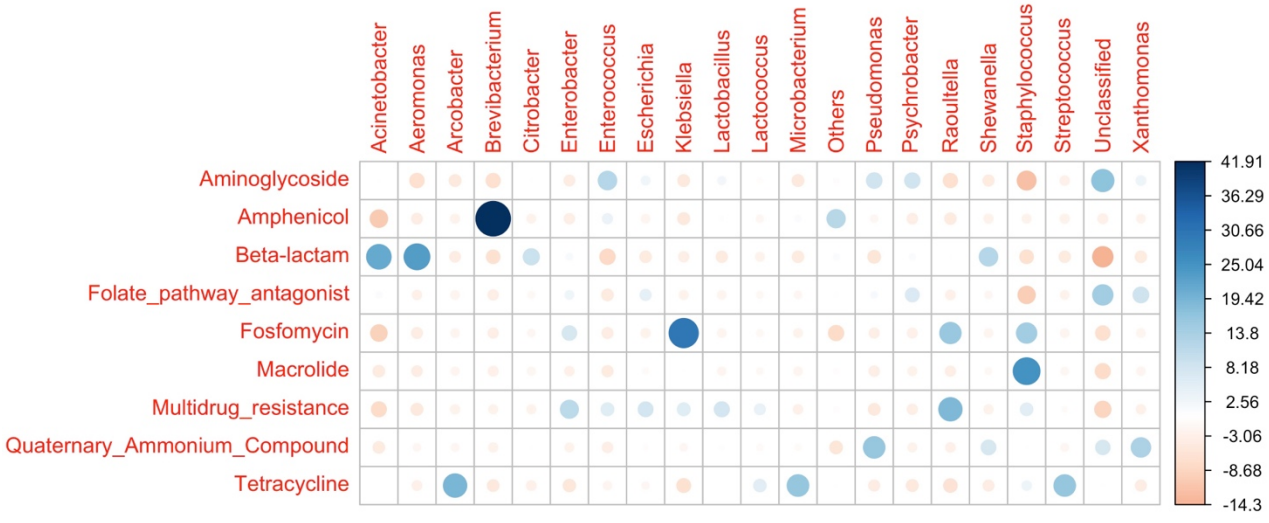

Figure 6.2 Plot of the χ^2 (chi-squared) Pearson residual. Blue circles indicate a positive correlation between the antibiotic (row) and the taxon (column).

6.3.2. The food industry resistome is highly mobilized

In order to understand whether the resistome is mobilized, we computed the percentage of positive calls for each class of genes according to the results from each tool (Figure 6.3). Quaternary Ammonium Compounds (QAC) genes were frequently associated to plasmids (82.2 % of the genes according to Platon), together with Aminoglycosides (72.45 %), Tetracyclines (54.15 %) and multidrug resistance genes (33.73 %). Also, 3% of the QAC resistance genes were predicted to be involved into LTG events, some of which involved *Staphylococcaceae* and *Lactobacillaceae*.

6.3.3. Multiple resistance genes are harbored on the same mobilized contigs

Furthermore, we checked whether some genes co-occurred on the same contigs. Therefore, we found 11 contigs hosting at least 4 ARGs (Figure 6.4). In particular, we observed that aminoglycoside resistance genes aph(3')-Ib and ant(3'')-Ia usually co-occurred together, and also

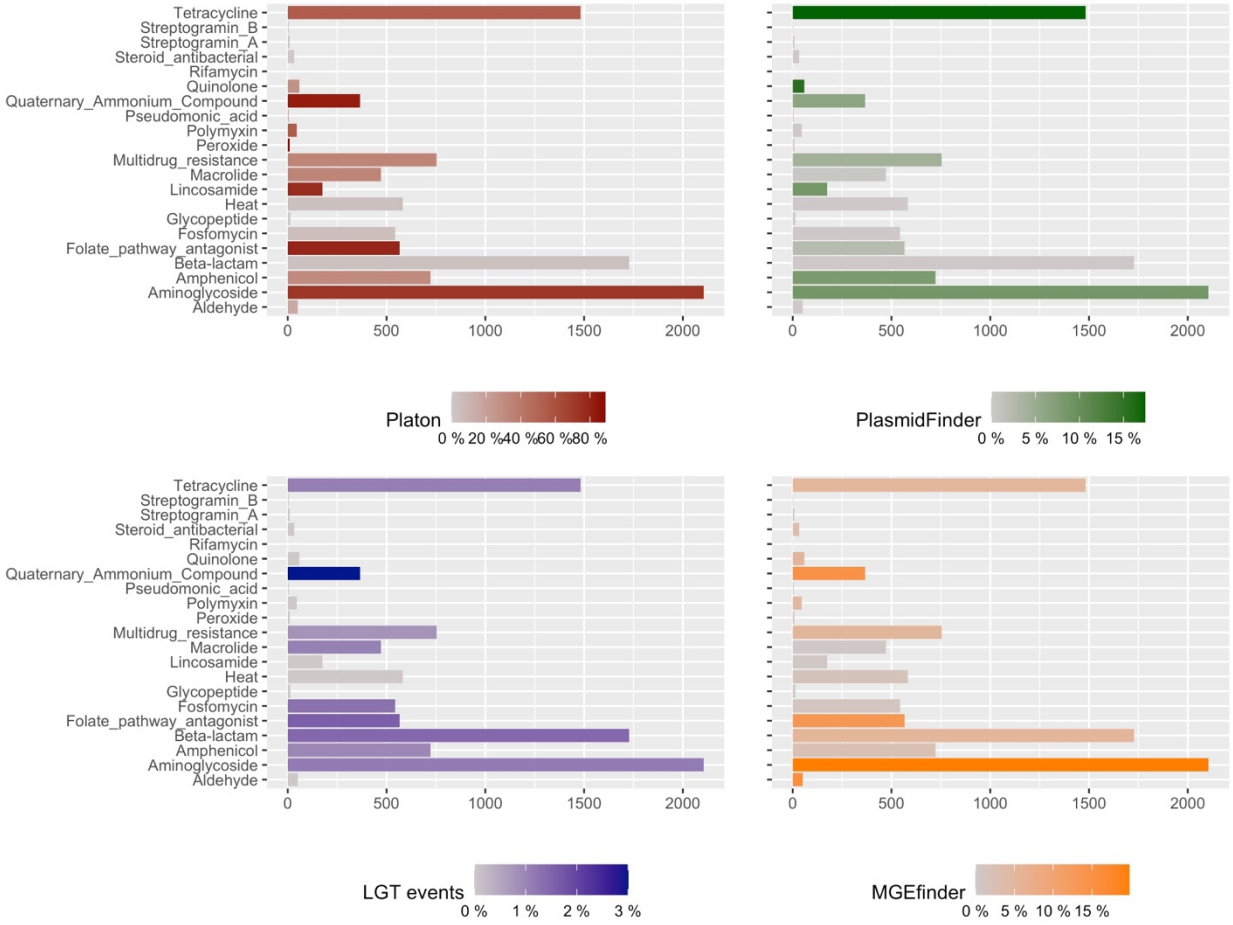

Figure 6.3 For each tool, the percentage of genes for each class receiving a positive call is reported.

with folate pathway antagonists (sul1 and sul2) and QAC genes (qacE). However, although we only focused on contigs showing at least 4 AR genes, more contigs showing multiple genes existed, with 169 and 963 contigs harboring at least 3 and 2 AR genes, respectively. Interestingly, all the contigs showing co-occurrence of genes were predicted to be part of plasmids.

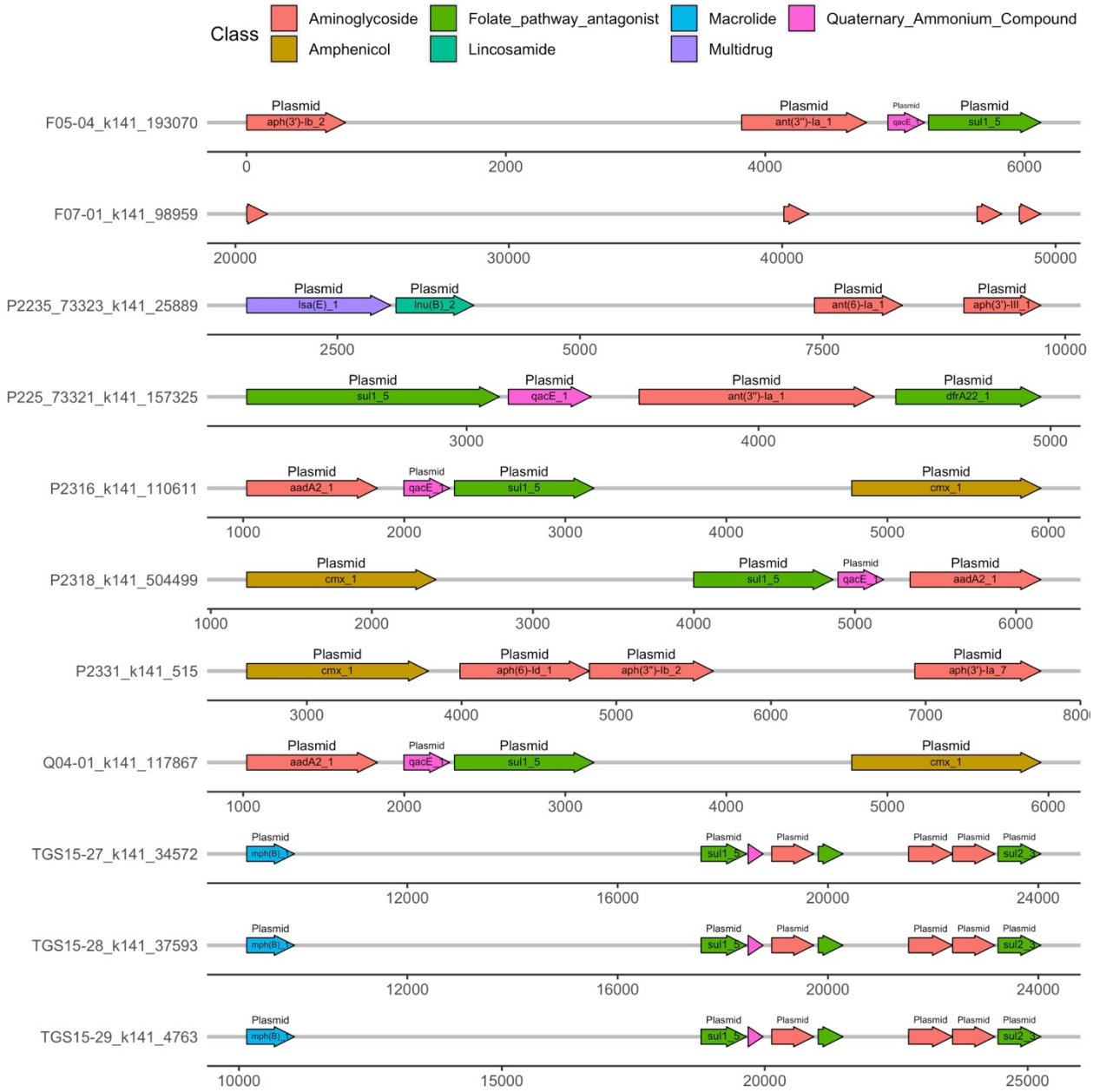

Figure 6.4 Contigs harboring ≥ 4 antibiotic resistance genes. Genes are color coded according to the class of resistance.

6.3.4. Read-level analysis

CPM for each gene were calculated. According to principal coordinate analyses based on the Bray-Curtis distance, no clear separation of samples according to the industry types emerge, except for the final products, that show a separation of cheeses from the other samples (Figure 6.5).

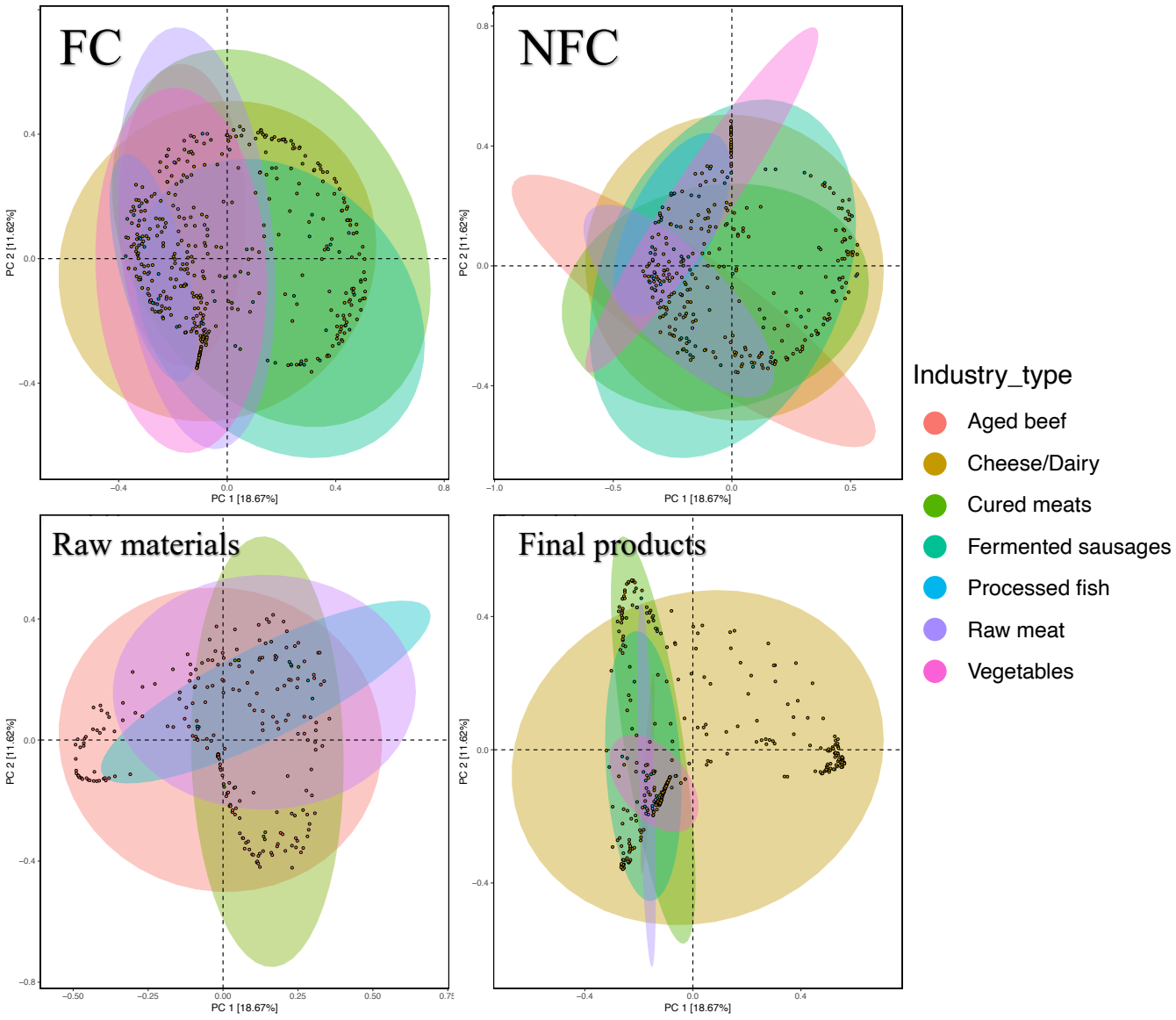

Figure 6.5 PCoA of the CPM abundance of the ARGs based on the Bray-Curtis distance.

In addition, read-level analysis also showed a higher abundance of AR genes on surfaces (both FC and NFC) than on ingredients, in all the facility types (Figure 6.6A). However, when comparing FC and NFC across all the industry types, facilities involved in processing of meats (cured, fermented and raw meats) evidently outstood the others (Figure 6.6B).

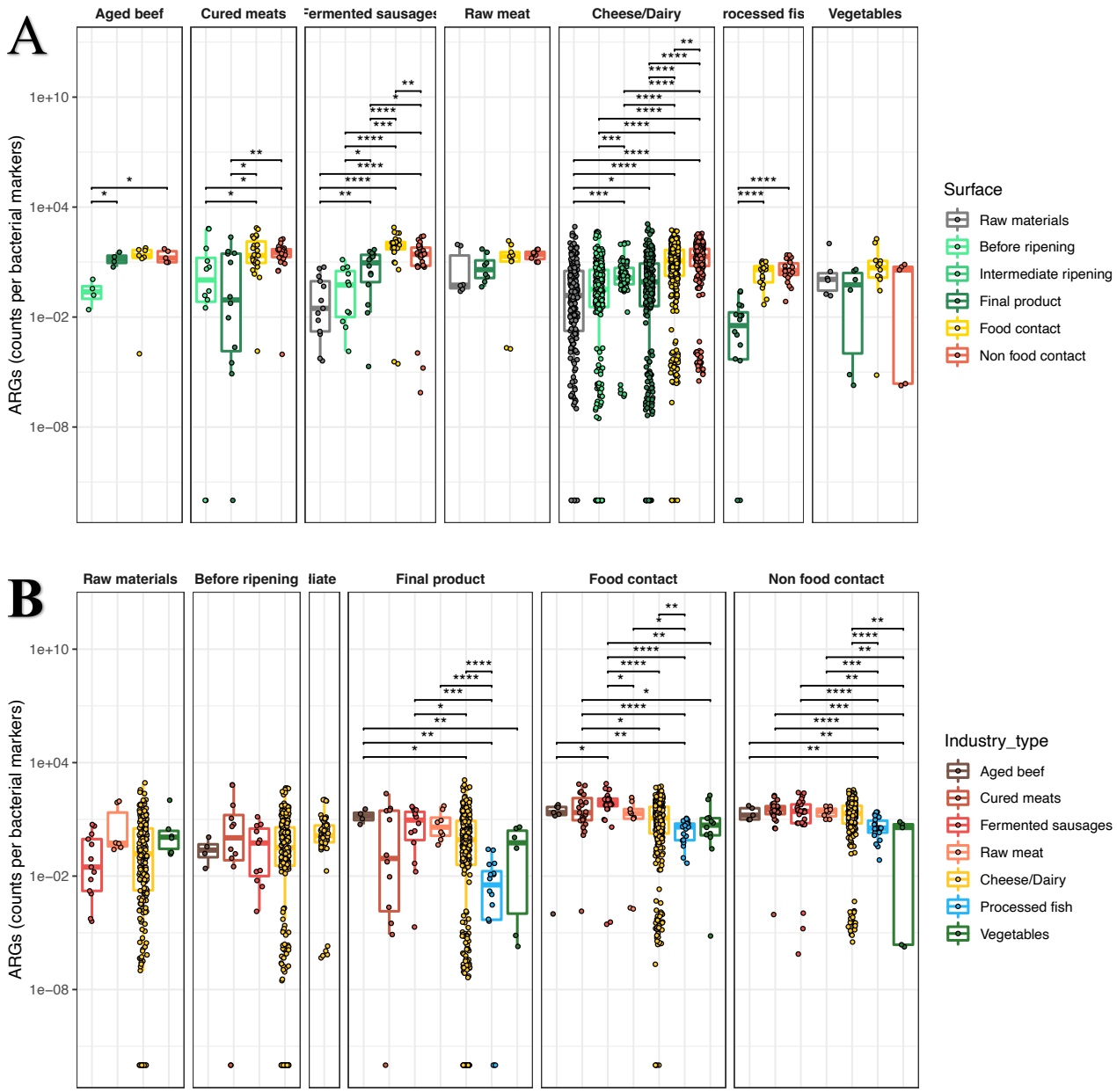

Figure 6.6 Boxplots comparing the CPM abundance of ARGs between the samples categories in the same facility type (A), or between the same samples group across multiple facilities (B).

6.4. Discussion

The food production chain may act as a reservoir of antibiotic-resistant microorganisms (Oniciuc et al., 2019), which might pose a problem for the human health because of transfer events from foods to human microorganisms (Singh et al., 2019).

Our results confirm the need to address this problem, since more than 50% of the samples collected in food processing facilities showed at least one ARG. Most of the genes are linked to *Acinetobacter*, *Staphylococcus* and *Enterococcus* spp. (Figure 6.1). This result is not surprising:

several authors reported the AR potential of these three clades, as well as their association with food industry. For example, *Acinetobacter* spp. have been often associated with dairy products (Gurung et al., 2013; Ramos et al., 2019) and meat (Klotz et al., 2018) and, although *A. baumannii* is the principal pathogen described within this species, antibiotic resistance has been reported for the whole genus (Crippen et al., 2020; Bello-López et al., 2020). Similarly, Wang and colleagues (2019) isolated more than 700 *Staphylococcus* spp. from Ready-To-Eat food, with the 85% of these showing resistance to at least one antibiotic, whereas Touimi et al. (2019) detected antibiotic resistance *Staphylococci* in 33% of the food contact surfaces they tested.

Our results suggest that *Acinetobacter* spp. are strongly linked to resistance to beta-lactams. This class of antibiotics, which also include penicillin, methicillin and cephalosporin, inhibits the cell wall synthesis, causing bacterial lysis (Fernandes et al., 2013). It is reported that several *Acinetobacter* possess a genomic island into their chromosome which codifies for more than 40 AR genes, some of which are active against carbapenem and oxacillines (Wong et al., 2017), which are widely used (and for some infections the sole option) to contrast both Gram-positive and Gram-negative bacteria (Wong et al., 2017).

Although most of the studies addressing the AR of *Staphylococcaceae* focus on Methicillin-Resistant *Staphylococcus aureus* (MRSA; Rodríguez-López et al., 2020; da Silva et al., 2019), we found a strong correlation between *Staphylococcus* spp. and macrolides-resistance genes (Figure 6.2). Consistently, Martini et al (2017) isolated about 90 strains of *Staphylococcus* spp. from milk samples, showing that > 70% of them were resistant to macrolides, tetracyclines and beta-lactams. Food industry stakeholders should pose a severe attention on resistance to tetracyclines, since they are among the most used antibiotics in the food industry (Granados-Chinchilla & Rodríguez, 2017).

Also, as a general trend, we observed that ARGs were more abundant and more diverse on FC surfaces than in foods (Figure 6.5A). One cause of such phenomenon is adherence of bacteria on abiotic surfaces through production of biofilms (Carrascosa et al., 2021). Biofilm production is an

excellent survival strategy, since bacteria are protected from antimicrobials and only exposed to sub-Minimum Inhibitor Concentrations (MIC) of such compounds (Zhang et al., 2020). *Acinetobacter* and *Staphylococcus* are able to produce biofilm in the food industry (de Souza et al., 2014; Nikolaev et al., 2022). In addition, the use of some compounds (such as disinfectants) to contrast biofilms might enhance antibiotic resistance through a mechanism called as cross-resistance (Colclough et al., 2019; Kampf, 2018). Also, horizontal gene transfer events are enhanced into biofilms (Madsen et al., 2012). For all these reasons, biofilms are considered a hot spot of antibiotic resistance gene development and transmission (Uruén et al., 2020), therefore food industry should develop alternative methods to contrast their attachment and maturation.

In addition, read-level analysis suggests that facilities producing meat (cured meat, aged beef and fermented meat) show a higher abundance of ARGs compared to other industry types (Figure 6.5B). This might be explained by the extended use of antibiotics in the animal sectors, accounting for ~80% of the total in some countries (WHO, 2017).

Also, we were not able to detect the taxonomic lineage of several contigs harboring ARGs. This suggests that there might be some species never described or isolated that contribute to AR. Interestingly, contigs from unclassified species were associated with aminoglycosides. This class of ARGs are very frequently found on plasmids and other mobile elements (Yang & Hu, 2022). Therefore, given the rate with which AR arises and is transferred, it can't be excluded that new uncharacterized species might have acquired these genes.

Our results suggest that a high percentage of aminoglycosides, QAC and tetracyclines resistance genes are encoded in plasmids or other mobile elements (Figure 6.3), with genes belonging to these three classes often co-occurring on the same contig (Figure 6.4). Even though these genes are not associated with pathogenic taxa, the high degree of mobilization of the ARGs still needs attention. In fact, it has been suggested that sharing the same ecological habitat is more important than phylogenetic distance in terms of HGT events regulation (Smillie et al., 2011). Therefore,

during the transit of food contaminated with AR species in the gut, HGT events between phylogenetically distant species might occur, as it has also been described before (Rolain, 2013). The fact that several ARGs are coded on the same MGE is not surprising, since the biological purpose of these genetic structures is to expand the host's ecological niche (Rodríguez-Beltrán et al., 2021). However, their existence on FC surfaces and in foods is concerning, since they might transmit multiple AR genes at once to, conferring resistance to multiple classes to microorganisms commonly eaten with food.

Taken collectively, these results indicate that AR genes are widespread in the food processing environment of several production areas, especially on surfaces in contact with foods, with *Acinetobacter* spp. and *Staphylococcus* spp. being the main carriers of resistance. However, although the high relevance and frequency of detection of these species, the high degree of mobilization of some classes of ARGs might promote HGT events with other microorganisms. It was previously suggested that sanitation procedure might exacerbate antibiotic resistance, therefore food industry should take all these information into account to develop novel methods to disinfect the food manufacture.

However, this work suffers from some weaknesses. Indeed, we only have information about genomic DNA, therefore we don't have data about expression of these genes. In addition, although whole metagenomic sequencing is a high-resolution approach that leads to detection and identification of species and genes, we might underestimate the AR potential of our metagenomes because of technological limitations related to short-reads assembly.

Therefore, further research involving metatrascriptomic and longer reads might be useful to better assess and characterize the food industry-associated mobilome.

6.5. References

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Conclusions

To ensure quality and safety of food, good hygiene standards should be maintained in the food industry. However, despite the efforts in development of aggressive cleaning and disinfection procedures, complex microbial communities still inhabit the food processing environment. In depth description of the taxonomic composition and of the metabolic potential of these microbes are needed in order to understand whether they are desirable or hazardous.

To face this, several strategies have been adopted, mainly relying on cultural methods. In this thesis, the potential of metagenomics was applied to explore the microbial communities coping with detergents and disinfectants in the food industry. A very high biodiversity of communities inhabiting the surfaces was observed, which was comparable to that of ingredients and products in the case of minimally processed vegetables. In addition, high quality MAGs were reconstructed from surfaces, thus suggesting their establishment in the food processing environment.

Some of the microorganisms found on surfaces harbored several genes associated with antibiotic resistance and with adherence and biofilm formation, as reported in Chapters 3, 4 and 6. In addition, most of these genes were reported to be part of plasmids or other mobile elements. Antibiotic resistance is a public health priority, which is estimated to cause more than 35,000 deaths in the EU (ECDC, 2022), and resistant strains from the environment might be transferred to the food product, thus representing a health hazard. Food business operators should be aware of these events and ensure safety of foods, developing novel procedures that aim at reducing these taxa or limiting factors potentially enhancing the spread of ARGs.

However, in some food industries, residential microbes might be advantageous. As observed in Chapter 5, cheesemaking facilities are inhabited by several Lactic Acid Bacteria species, which might not only contrast the establishment of pathogens through the production of several bacteriocins, but also contribute to shape the sensorial profile of cheeses, making it unique.

In general, mapping the environmental microbiome in food facilities revealed new insights into communities'structure, dynamics and metabolic potential of microbes residing on surfaces, also

leading to identification of virulence factors that help microbes to establish on food contact surfaces. Also, the procedure was useful to rapidly identify putative pathogens and genes linked with pathogenesis, as observed in chapter 3. Therefore, the procedure might support quality and safety management plans in the near future, also helping food business operators to reduce spoilage-caused food loss and make the food industry more sustainable.

However, there are some critical points to address before their adoption as routine practices. Indeed, the environmental mapping described in this thesis is based on DNA sequencing. These data only depict the metabolic potential of these microbes, and they do not provide information about the ongoing biochemical processes.

In addition, integration of these procedures in the food industry is still limited by the lack of bioinformatics skills, which are necessary to analyze and interpret data, and by the cost of sequencing that, although constantly decreasing, might represent a hurdle for small and medium-sized companies.

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List of publications

Publications included in the thesis

1. De Filippis, F., **Valentino, V.**, Alvarez-Ordóñez, A., Cotter, P. D., & Ercolini, D. (2021). Environmental microbiome mapping as a strategy to improve quality and safety in the food industry. *Current Opinion in Food Science, 38,* 168-176. https://doi.org/10.1016/j.cofs.2020.11.012.
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