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# 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.

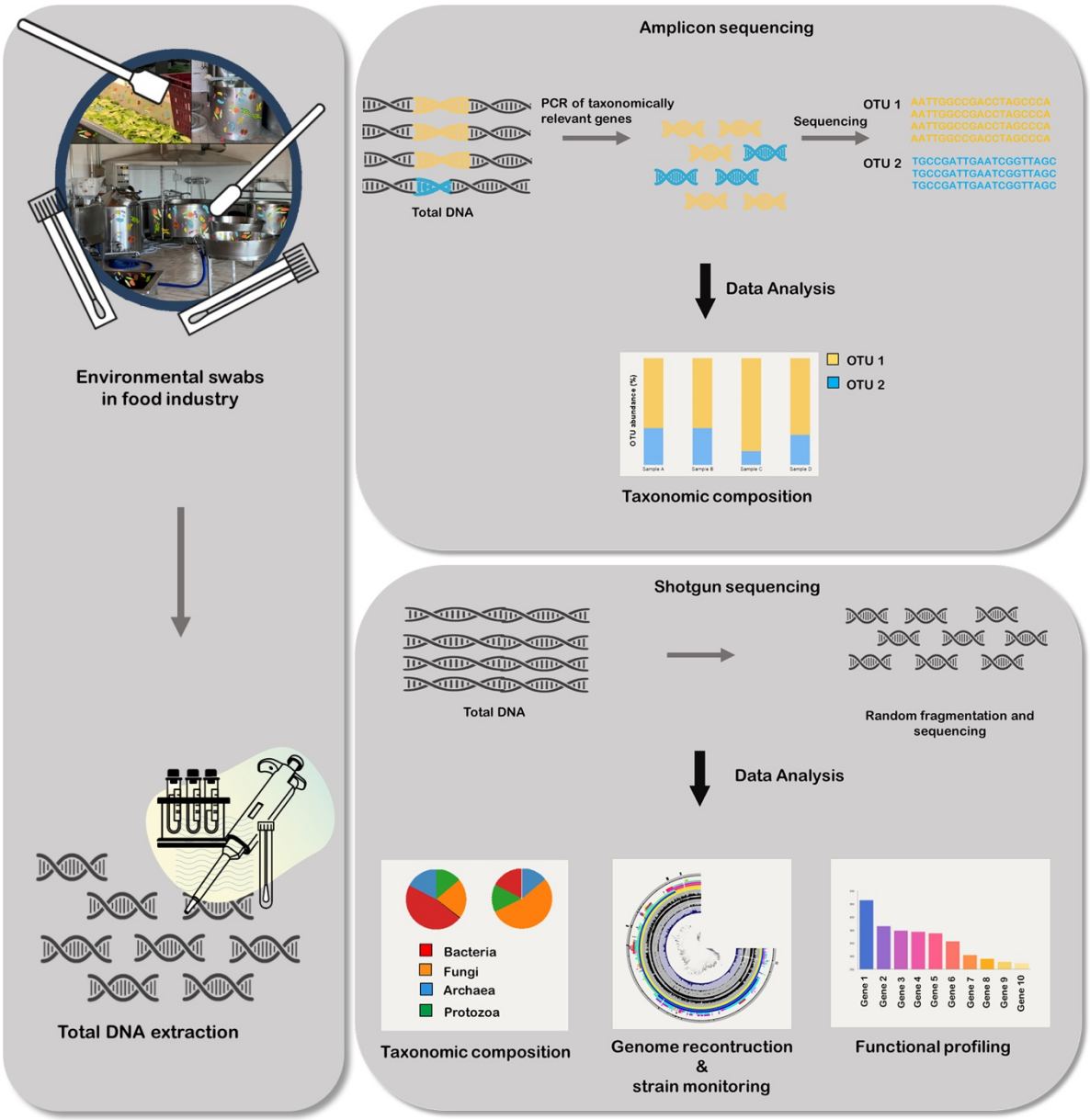


**Figure 2.2** High-throughput sequencing approaches for microbiome mapping. Different high-throughput sequencing approaches for the study of environmental microbiome in food industry.

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.

### *2.7. References*


1. Alexa, O. E. A., Walsh, C. J., Coughlan, L. M., Awad, A., Simon, C. A., Ruiz, L., Crispie, F., Cotter, P. D., & Alvarez-Ordóñez, A. (2020). Dairy products and dairy-processing environments as a reservoir of antibiotic resistance and quorum-quenching determinants as revealed through functional metagenomics. *mSystems*, *5(1)*, e00723-19. https://doi.org/10.1128/mSystems.00723-19.
2. Araújo, E. A., de Andrade, N. J., Mendes da Silva, L.H., de Carvalho, A. F., de Sá Silva, C. A., & Ramos, A. M. (2010). Control of microbial adhesion as a strategy for food and bioprocess technology**.** *Food and Bioprocess Technology*, *3*, 321-332.
3. Bokulich, N. A., Bergsveinson, J., Ziola, B., Mills, D. A. (2015). Mapping microbial ecosystems and spoilage-gene flow in breweries highlights patterns of contamination and resistance. *eLife*, *4*, e04634. https://doi.org/e04634.10.7554/eLife.04634.
4. Bokulich, N. A., Mills, D. A. (2013). Facility-specific "house" microbiome drives microbial landscapes of artisan cheesemaking plants. *Applied Environmental Microbiology*, *79(17)*, 5214-5223.
5. Bokulich, N. A., Ohta, M., Lee, M., & Mills, D. A. (2014). Indigenous Bacteria and Fungi drive traditional Kimoto sake fermentations. *Applied Environmental Microbiology*, *80(17)*, 5522-5529.
6. Bokulich, N. A., Ohta, M., Richardson, P. M., & Mills, D. A. (2013). Monitoring seasonal changes in winery-resident microbiota. *PLoS One*, *8(6)*, e66437. https://doi.org/10.1371/journal.pone.0066437.
7. Bruijns, B. B., Tiggelaar, R. M., & Gardeniers, H. (2018). The extraction and recovery efficiency of pure DNA for different types of swabs. *Journal of Forensic Science*, *63(5)*,1492-1499.

8. Cai, L., Wu, D., Xia, J., Shi, H., & Kim, H. (2019). Influence of physicochemical surface properties on the adhesion of bacteria onto four types of plastics. *Science of Total Environment*, *671*, 1101-1107.

9. Calasso, M., Ercolini, D., Mancini, L., Stellato, G., Minervini, F., Di Cagno, R., De Angelis, M., Gobetti, M. (2016). Relationships among house, rind and core microbiotas during manufacture of traditional Italian cheeses at the same dairy plant. *Food Microbiology*, *54*, 115-126.

10. Castellano, P., Pérez Ibarreche, M., Blanco Massani, M., Fontana, C., & Vignolo, G. M. (2017). Strategies for pathogen biocontrol using lactic acid bacteria and their metabolites: A focus on meat ecosystems and industrial environments microorganisms. *Microorganisms*, *5(3)*, 38. https://doi.org/10.3390/microorganisms5030038.

11. Dalmaso G., Bini, M., Paroni, R., & Ferrari, M. (2008). Qualification of high-recovery, flocked swabs as compared to traditional rayon swabs for microbiological environmental monitoring of surfaces. *PDA Jorunal of Pharmaceutical Science and Technology*, *62(3)*, 191-199.

12. De Filippis, F., La Storia, A., Villani, F., & Ercolini, D. (2013). Exploring the sources of bacterial spoilers in beefsteaks by culture-independent high-throughput sequencing. *PLoS One*, *8(7)*, e70222. https://doi.org/10.1371/journal.pone.0070222.

13. De Filippis, F., Laiola, M., Blaiotta, G., & Ercolini, D. (2017). Different amplicon targets for sequencing-based studies of fungal diversity. *Applied Environmental Microbiology*, *83(17)*, e00905-17.

14. De Filippis, F., Parente, E., & Ercolini, D. (2018). Recent past, present, and future of the food microbiome. *Annual Reviews of Food Science and Technology*, *9*, 589-608.

15. De los Mozos, E. A., Badurdeen, F., & Dossou, P. E. (2020). Sustainable Consumption by Reducing Food Waste: a Review of the Current State and Directions for Future Research. *Procedia Manufacturing, 51,* 1791-1798. https://doi.org/10.1016/j.promfg.2020.10.249.

16. Dzieciol, M., Schornsteiner, E., Muhterem-Uyar, M., Stessl, B., Wagner, M., & Schmitz-Esser, S. (2016). Bacterial diversity of floor drain biofilms and drain waters in a *Listeria monocytogenes* contaminated food processing environment. *International Journal of Food Microbiology*, *223,* 33-40.

17. EFSA Panel on Biological Hazards (2019). Whole genome sequencing and metagenomics for outbreak investigation, source attribution and risk assessment of food-borne microorganisms. *EFSA Journal*, *17(12)*, e05898.

18. Falardeau, J., Keeney, K., Trmčić, A., Kitts, D., Wang, S. (2019). Farm-to-fork profiling of bacterial communities associated with an artisan cheese production facility. *Food Microbiology*, *83*, 48-58.

19. Griffith, C. (2005). Improving surface sampling and detection of contamination. In: *Handbook of hygiene control in the food industry*. Edited by Lelieveld H, Holah J, Mostert M. Woodhead Publishing; 588-618.

20. Guzzon, R., Carafa, I., Tuohy, K., Cervantes, G., Vernetti, L., Barmaz, A., Larcher, R., & Franciosi, E. (2017). Exploring the microbiota of the red-brown defect in smear-ripened cheese by 454-pyrosequencing and its prevention using different cleaning systems. *Food Microbiology*, *62*, 160-168.

21. Hultman, J., Rahkila, R., Ali, J., Rousu, J., & Björkroth, K. J. (2015). Meat processing plant microbiome and contamination patterns of cold-tolerant bacteria causing food safety and spoilage risks in the manufacture of cacuum-packaged cooked sausages. *Applied Environmental Microbiology*, *81(20)*, 7088-7097.

22. Kable, M. E., Srisengfa, Y., Laird, M., Zaragoza, J., McLeod, J., Heidenreich, J., & Marco, M. L. (2016). The core and seasonal microbiota of raw bovine milk in tanker trucks and the impact of transfer to a milk processing facility. *mBio*, *7(4)*, e00836-16. https://doi.org/10.1128/mBio.00836-16.

23. Kable, M. E., Srisengfa, Y., Xue, Z., Coates, L. C., & Marco, M. L. (2019). Viable and total bacterial populations undergo equipment- and time- dependent shifts during milk processing. *Applied Environmental Microbiology*, *85(13),* e00270-19. https://doi.org/10.1128/AEM.00270-19.

24. Kim, K. H., & Bae, J. W. (2011). Amplification methods bias metagenomic libraries of uncultured single-stranded and double-stranded DNA viruses. *Applied Environmental Microbiology*, *77(21)*, 7663-7668.

25. McHugh, A. J., Feehily, C., Fenelon, M. A., Gleeson, D., Hill, C., Cotter, P. D. (2020). Tracking the dairy microbiota from farm bulk tank to skimmed milk powder. *mSystems*, *5(2)*, e00226-20. https://doi.org/10.1128/mSystems.00226-20.

26. Midha, M. K., Wu, M., & Chiu, K. (2019). Long-read sequencing in deciphering human genetics to a greater depth. *Human Genetic*, *138(11-12)*, 1201-1215.

27. Minervini, F., Lattanzi, A., De Angelis, M., Celano, G., & Gobetti, M. (2015). House microbiotas as sources of lactic acid bacteria and yeasts in traditional Italian sourdoughs. *Food Microbiology, 52*, 66-76.

28. Møretrø, T., & Langsrud, S. (2017). Residential bacteria on surfaces in the food industry and their implications for food safety and quality. *Comprehensive Reviews in Food Science and Food Safety*, *16(5)*, 1022-1041.

29. Møretrø, T., Moen, B., Heir, E., Hansen, A. A., & Langsrud, S. (2016). Contamination of salmon fillets and processing plants with spoilage bacteria. *International Journal of Food Microbiology*, *237*, 98-108.

30. Motz, V. A., Young, L. M., Motz, M. E., & Young, S. C. (2019). A Sticking point in assessing bacterial contamination: Adhesive characters of bacterial specializations, swab features, and fomite surface properties skew colony counts. *Journal of Pure and Applied Microbiology*, *13(4)*, 2533-2544.

31. Pang, X. N., Han, B. Z., Huang, X. N., Zhang, X., Hou, L. F., Cao, M., Gao, L. J., Hu, G. H., & Chen, J. Y. (2018). Effect of the environment microbiota on the flavour of light-flavour Baijiu during spontaneous fermentation. *Scientific Reports*, *8(1)*, 3396. https://doi.org/10.1038/s41598-018-21814-y.

32. Parker, M., Zobrist, S., Donahue, C., Edick, C., Mansen, K., Hassan Zade Nadjari, M., Heerikhuisen, M., Sybesma, W., Molenaar, D., Diallo A. M., *et al*. (2018). Naturally fermented milk from Northern Senegal: Bacterial community composition and probiotic enrichment with *Lactobacillus rhamnosus*. *Frontiers in Microbiology,* 9, 2218. https://doi.org/10.3389/fmicb.2018.02218.

33. Pothakos, V., Stellato, G., Ercolini, D., & Devlieghere, F. (2015). Processing environment and ingredients are both sources of *Leuconostoc gelidum*, which emerges as a major spoiler in ready-to-eat meals. *Applied Environmental Microbiology*, *81(10)*, 3529-3541.

34. Quijada, N. M., Mann, E., Wagner, M., Rodríguez-Lázaro, D., Hernández, M., & Schmitz-Esser, S. (2018). Autochthonous facility-specific microbiota dominates washed-rind Austrian hard cheese surfaces and its production environment. *International Journal of Food Microbiology, 267*, 54-61.

35. Schön, K., Schornsteiner, E., Dzieciol, M., Wagner, M., Müller, M., & Schmitz-Esser, S. (2016). Microbial communities in dairy processing environment floor-drains are dominated by product-associated bacteria and yeasts. *Food Control*, *70*, 210-215.

36. Son, H., Park, S., Beuchat, L. R., Kim, H., & Ryu, J. H. (2016). Inhibition of *Staphylococcus aureus* by antimicrobial biofilms formed by competitive exclusion microorganisms on stainless steel. *International Journal of Food Microbiology*, *238*, 165-171.

37. Stellato, G., De Filippis, F., La Storia, A., Ercolini, D. (2015). Coexistence of lactic acid bacteria and potential spoilage microbiota in a dairy-processing environment. *Applied Environmental Microbiology*, *81(22)*, 7893-7904.

38. Stellato, G., La Storia, A., De Filippis, F., Borriello, G., Villani, F., & Ercolini, D. (2016). Overlap of spoilage-associated microbiota between meat and meat processing environment in small-scale and large-scale retail distribution. *Applied Environmental Microbiology*, *82(13)*, 4045-4054.

39. Tan, X., Chung, T., Chen, Y., Macarisin, D., LaBorde, L., & Kovac, J. (2019). The occurrence of *Listeria monocytogenes* is associated with built environment microbiota in three tree fruit processing facilities. *Microbiome*, *7(1)*, 115. https://doi.org/10.1186/s40168-019-0726-2.

40. Verdon, T. J., Mitchell, R. B., & van Oorschot, R. A. (2014). Swabs as DNA collection devices for sampling different biological materials from different substrates. *Journal of Forensic Science*, *59(4)*, 1080-1089.

41. Yilmaz, S., Allgaier, M., & Hugenholtz, P. Multiple displacement amplification compromises quantitative analysis of metagenomes. *Nature Methods*, *7(12)*, 943-944. doi:10.1038/nmeth1210-943.

42. Yokouchi, H., Fukuoka, Y., Mukoyama, D., Calugay, R., Takeyama, H., Matsunaga, T. (2006). Whole-metagenome amplification of a microbial community associated with scleractinian coral by multiple displacement amplification using phi29 polymerase. *Environmental Microbiology*, *8(7)*, 1155-1163.

43. Zasada, A. A., Zacharczuk, K., Woźnica, K., Główka, M., Ziółkowski, R., & Malinowska, E. (2020). The influence of a swab type on the results of point-of-care tests. *AMB Express*, *10(1)*, 46. https://doi.org/10.1186/s13568-020-00978-9.

# 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.

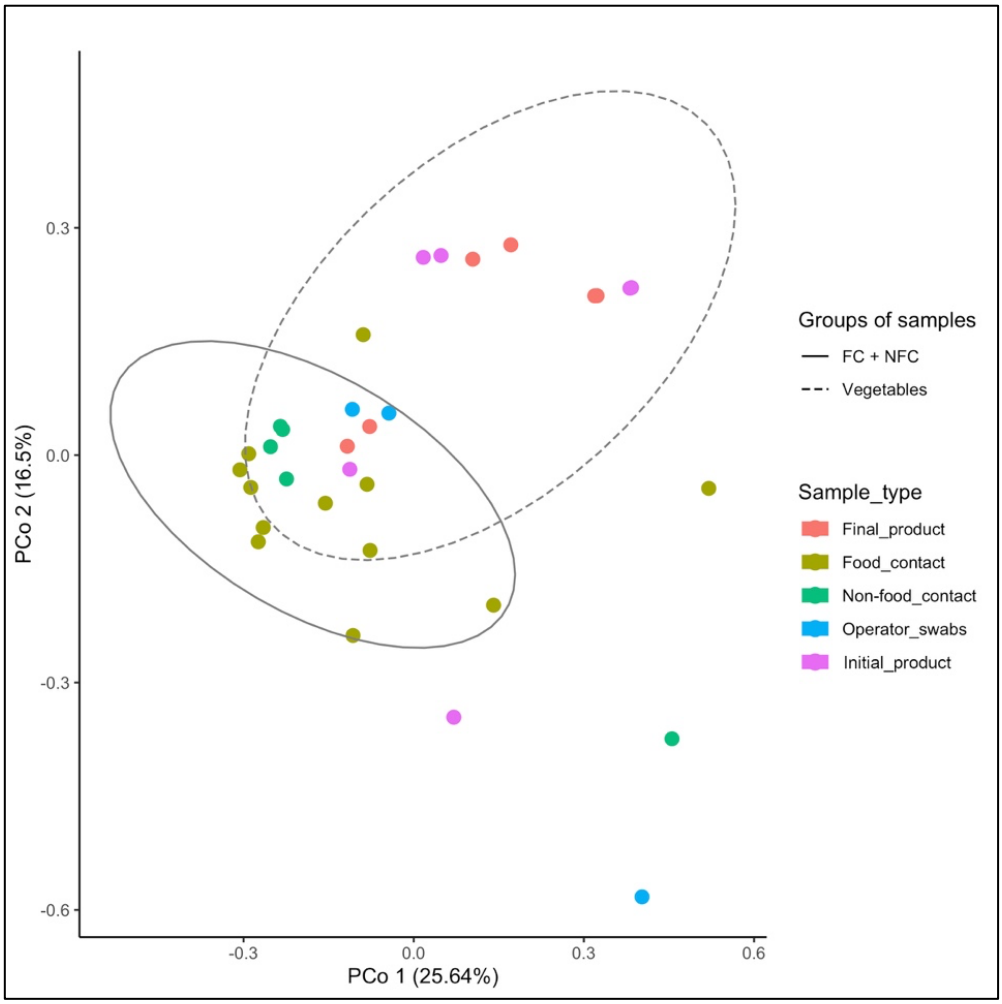


**Figure 3.1** PCoA based on the Bray-Curtis distance performed on the genus-level bacterial profiles obtained with Kraken2. Points are color-coded according to the sample type. Ellipses are drawn around surfaces (FC + NFC) and Vegetables (Initial + Final products).

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

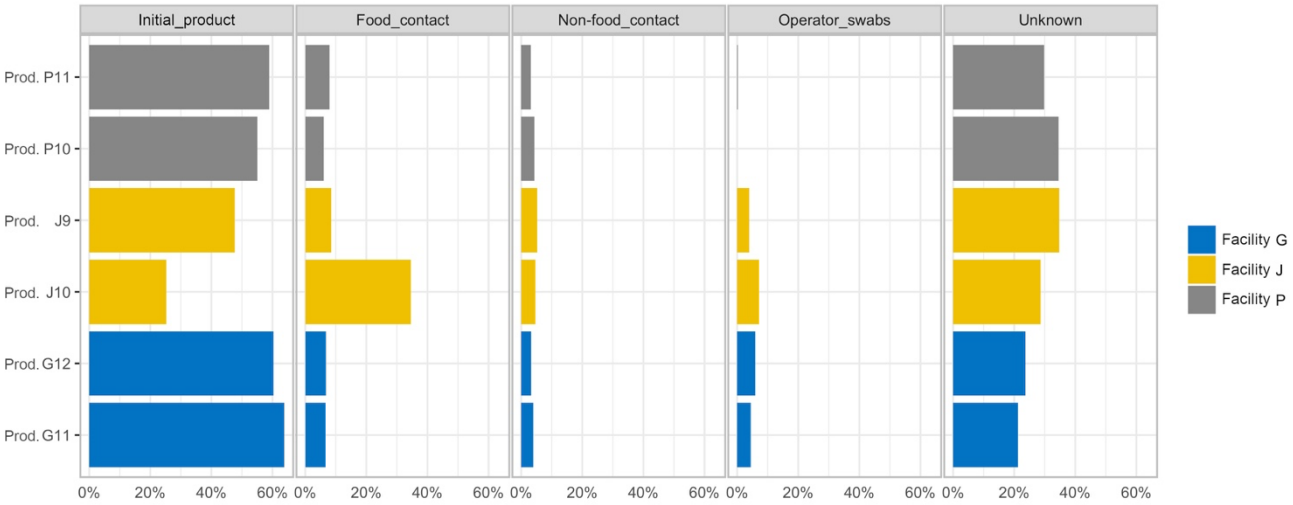


**Figure 3.2** Barplot showing the percentage contribution (x-axis) of each source of contamination to the taxonomic composition of the final products (y-axis). The 2 final products from each facility were analysed independently.


**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.

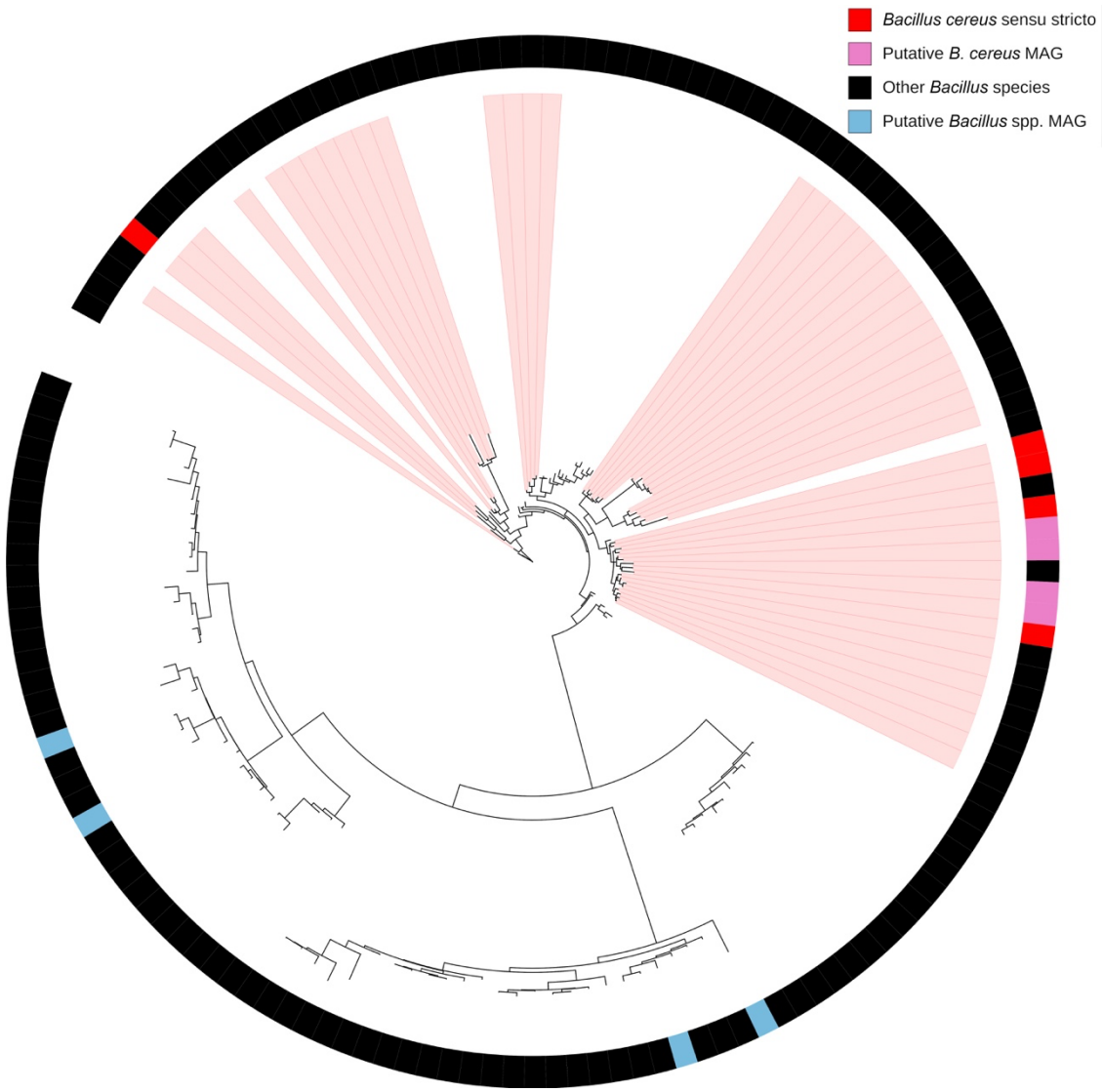


**Figure 3.4** Phylogenetic tree of a subset of NCBI RefSeq genomes spanning across multiple *Bacillus* species and those MAGs from surfaces and vegetables attributed to *Bacillus*. Clades highlighted in red belong to the *Bacillus cereus* group.

*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.

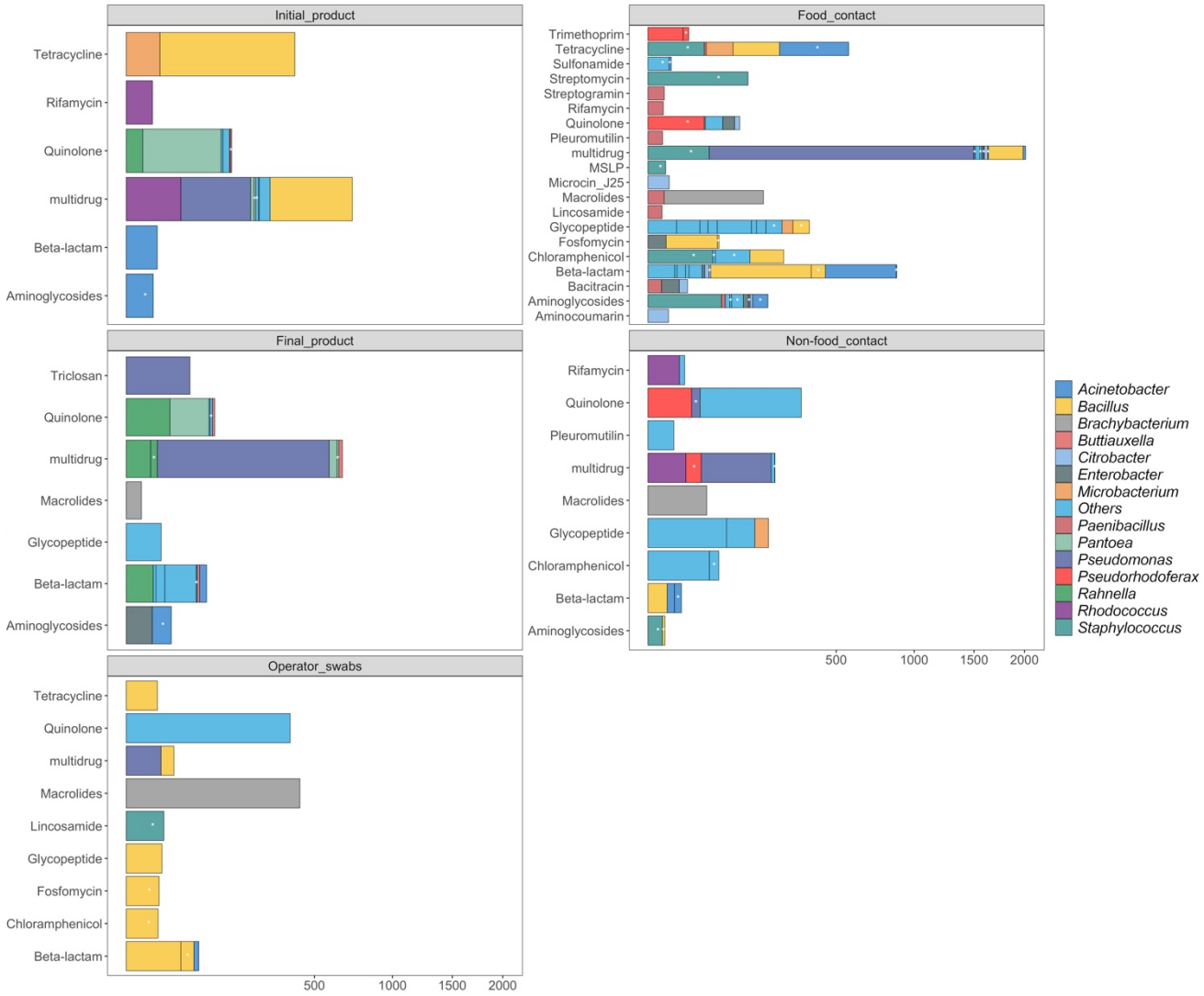


**Figure 3.5** Barplot showing, for each sample category, the abundance in RPKM of the Antibiotic Resistance Genes classes. Bars are color-coded according to the taxonomic assignment of the ARG-carrying contigs reported by Kraken2. Genes marked with an asterisk (*) are reported to be part of plasmids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

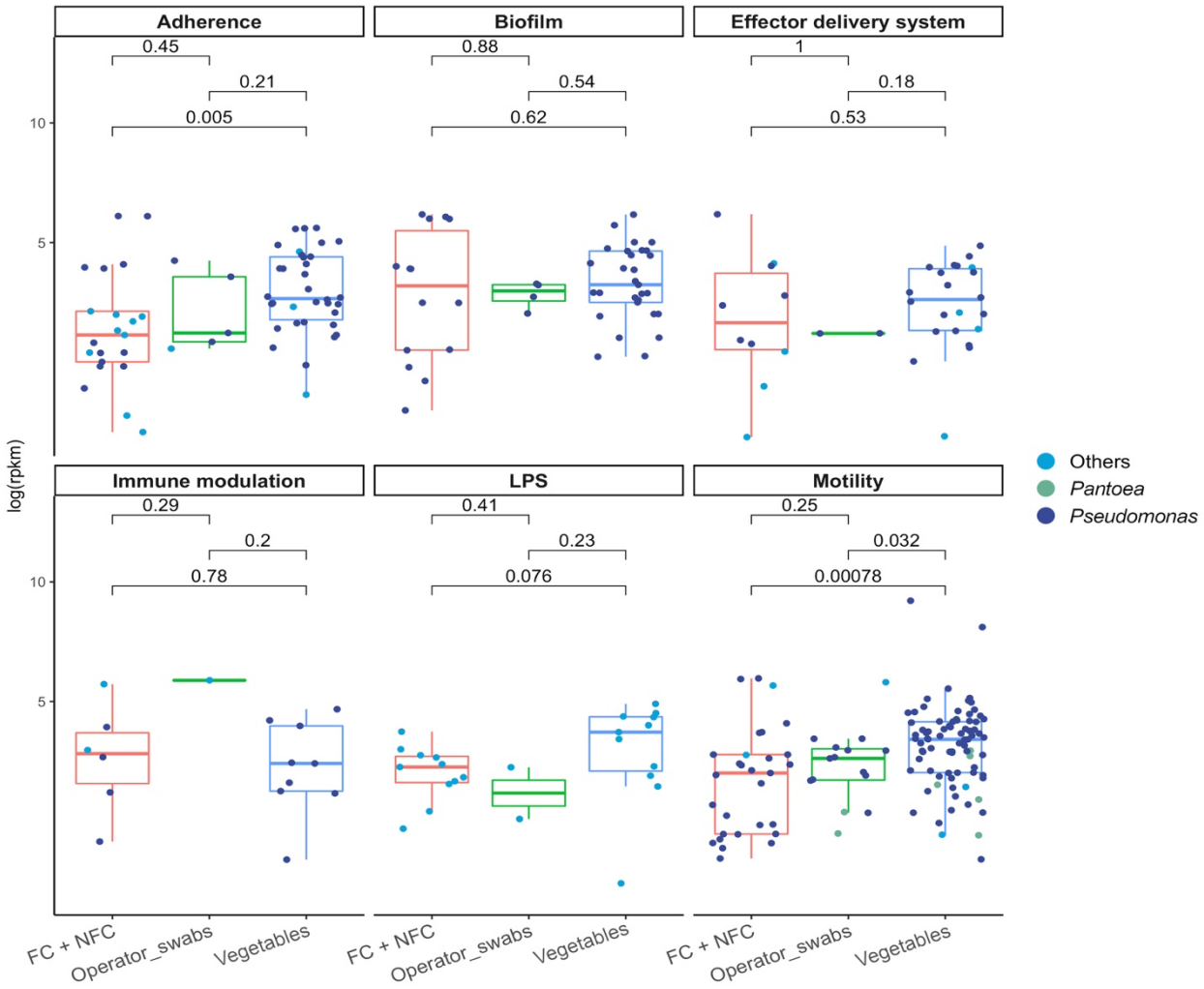


**Figure 3.6** Boxplot showing the RPKM abundance (in log scale) of several Virulence Factor Genes (VFGs) for each group of samples ("FC+NFC", "Operator swabs" and "Vegetables"). Points are color-coded according to the taxonomic assignment of the VFG-carrying contigs reported by Kraken2.

### *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*


1. Afolabi, R. O., Oloyede, A. R. & Ibrahim, T. A. (2011). Evaluation of pathogenic bacteria associated with fresh produce obtained from selected markets in Abeokuta. *Journal of Science & Sustainable Development,* 4, 75–81. https://doi.org/10.4314/jssd.v4i1.7.
2. Al-Kharousi, Z. S., Guizani, N., Al-Sadi, A. M., & Al-Bulushi, I. M. (2016). Hiding in fresh fruits and vegetables: Opportunistic pathogens may cross geographical barriers. *International Journal of Microbiology*. Article 4292417. https://doi.org/10.1155/ 2016/4292417.
3. Bae, Y.-M., Zheng, L., Hyun, J.-E., Jung, K.-S., Heu, S., & Lee, S.-L. (2014). Growth characteristics and biofilm formation of various spoilage bacteria isolated from fresh produce. *Journal of Food Science, 79*(10), M2072–M2080. https://doi.org/10.1111/ 1750-3841.12644.
4. Barcenilla, C., Cobo-Diaz, J. F., Puente, A., Armanini, F., Carlino, N., Blanco-Míguez, A., Pinto, F., Cabrera Rubio, R., Quijada, N. M., Dziecio, M., O'Neil, D., Mahler de Sanchez, L., De Filippis, F., Valentino, V., Calvete-Torre, I., Sabater, C., Delgrado, S., Ruas-Madiedo, P., López, M., ... Álvarez-Ordóñez, A. Improved sampling and DNA extraction procedures for characterising the microbiome of food processing environments through whole metagenome sequencing. *Unpublished results*.

5. Blau, K., Bettermann, A., Jechalke, S., Fornefeld, E., Vanrobaeys, Y., Stadler, T., Top, E. M., & Smalla, K. (2018). The Transferable Resistome of Produce. *mBio, 9*(6), e01300–e01318.
6. Carstens, C. K., Salazar, J. K. & Darkoh, C. (2019). Multistate outbreaks of foodborne illness in the United States associated with fresh produce from 2010 to 2017. *Frontiers in Microbiology,* 10, 2667. doi: 10.3389/fmicb.2019.02667.
7. Carter, M. Q. & Brandl, M. T. (2015). Biofilms in fresh vegetables and fruits. In A. L. Pometto, & A. Demirci (Eds.), *Biofilm in the food environment.* John Wiley & Sons, Ltd.
8. Chaumeil, P., Mussig, A., Hugenholtz, P., & Parks, D. (2020). GTDB-Tk: A toolkit to classify genomes with the Genome Taxonomy Database. *Bioinformatics, 36*(6), 1925–1927. https://doi.org/10.1093/bioinformatics/btz848.
9. Chen, B., Han, J., Dai, H., & Jia, P. (2021). Biocide-tolerance and antibiotic-resistance in community environments and risk of direct transfers to humans: Unintended consequences of community-wide surface disinfecting during COVID-19? *Environmental Pollution, 283*, Article 117074. https://doi.org/10.1016/j.envpol.2021.117074.
10. Chen, L., Yang, J., Yao, Z., Sun, L., Shen, Y., & Jin, Q. (2005). VFDB: A reference database for bacterial virulence factors. *Nucleic Acids Research, 33*, D325–D328. https://doi.org/10.1093/nar/gki008.
11. Chen, L., Zou, Y., She, P., & Wu, Y. (2015). Composition, function, and regulation of T6SS in *Pseudomonas aeruginosa*. *Microbiology Research, 172*, 19–25. https://doi.org/10.1016/j.micres.2015.01.004.
12. da Silva, S. F., Reis, I. B., Monteiro, M. G., Dias, V. C., Machado, A. B. F., da Silva, V. L., & Diniz, C. G. (2021). Influence of human eating habits on antimicrobial resistance phenomenon: Aspects of clinical resistome of gut microbiota in omnivores, ovolactovegetarians, and strict vegetarians. *Antibiotics (Basel), 10*(3), 276. https://doi.org/10.3390/antibiotics10030276.

13. De Filippis, F., Valentino, V., Álvarez-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.

14. Deakin, G., Tilston, E. L., Bennett, J., Passey, T., Harrison, N., Fernández-Fernández, F., & Xu, X. (2018). Spatial structuring of soil microbial communities in commercial apple orchards. *Applied Soil Ecology, 130*, 1–12. https://doi.org/10.1016/j.apsoil.2018.05.015.

15. Fazli, M., Almblad, H., Rybtke, M. L., Givskov, M., Eberl, L., & Tolker-Nielsen, T. (2014). Regulation of biofilm formation in *Pseudomonas* and *Burkholderia* species. *Environmental Microbiology, 16*(7), 1961–1981. https://doi.org/10.1111/1462-2920.12448.

16. Fiedler, G., Schneider, C., Igbinosa, E. O., Kabisch, J., Brinks, E., Becker, B., Stoll, D. A., Cho, G.-S., Huch, M., & Franz, C. M. A. P. (2019). Antibiotics resistance and toxin profiles of *Bacillus cereus*-group isolates from fresh vegetables from German retail markets. *BMC Microbiology, 19*(1), 250. https://doi.org/10.1186/s12866-019-1632- 2.

17. Flores-Vargas, G., Bergsveinson, J., Lawrence, J., & Korber, D. (2021). Environmental biofilms as reservoirs for antimicrobial resistance. *Frontiers in Microbiology, 12*, Article 766242. https://doi.org/10.3389/fmicb.2021.766242.

18. Food and Drug Administration (2008). *Guidance for industry: Guide to minimize microbial food safety hazards of fresh-cut fruits and vegetables*. Retrieved from https://www.fda. gov/regulatory-information/search-fda-guidance-documents/guidance-industry-guide-minimize-microbial-food-safety-hazards-fresh-cut-fruits-and-vegetables/. Accessed June 1, 2022.

19. Founou, L. L., Founou, R. C., & Essack, S. Y. (2016). Antibiotic resistance in the food chain: A developing country-perspective. *Frontiers in Microbiology, 7*, 1881. https://doi.org/10.3389/ fmicb.2016.01881.

20. Ghaly, T.M., Chow, L., Asher, A. J., Waldron, L. S., & Gillings, M. R. (2017). Evolution of class 1 integrons: Mobilization and dispersal via food-borne bacteria. *PLoS ONE, 12* (6), e0179169. doi: 10.1371/journal.pone.0179169.

21. Guevarra, R. B., Magez, S., Peeters, E., Chung, M. S., Kim, K. H., & Radwanska, M. (2021). Comprehensive genomic analysis reveals virulence factors and antibiotic resistance genes in *Pantoea agglomerans* KM1, a potential opportunistic pathogen. *PLoS ONE, 16*(1), e0239792. https://doi.org/10.1371/journal.pone.0239792.

22. Guzm´an, A., Gonz´alez Hurtado, M., Cuesta-Astroz, Y., & Torres, G. (2020). Metagenomic characterization of bacterial biofilm in four food processing plants in Colombia. *Brazilian Journal of Microbiology, 51*(3), 1259–1267. https://doi.org/10.1007/s42770-020-00260-x

23. Isoken, H. I. (2015). Biofilm formation of *Salmonella* species isolated from fresh cabbage and spinach. *Journal of Applied Sciences and Environmental Management, 19*(1), 45. https://doi.org/10.4314/jasem.v19i1.6

24. Jiao, S., Xu, Y., Zhang, J., Hao, X., & Lu, Y. (2019). Core microbiota in agricultural soils and their potential associations with nutrient cycling. *mSystems, 4*(2), e00313–e00318. https://doi.org/10.1128/mSystems.00313-18

25. Jin, M., Liu, L., Wang, D.-N., Yang, D., Liu, W.-L., Yin, J., Yang, Z.-W., Wang, H.-R., Qiu, Z.-G., Shen, Z.-Q., Shi, D.-Y., Li, H.-B., Guo, J.-H., & Li, J.-W. (2020). Chlorine disinfection promotes the exchange of antibiotic resistance genes across bacterial genera by natural transformation. *The ISME Journal, 14*(7), 1847–1856. https://doi.org/10.1038/s41396-020-0656-9.

26. Kang, D., Froula, J., Egan, R. & Wang, Z. (2015). MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ, 3*, e1165. https://doi.org/10.7717/peerj.1165.

27. Knights, D., Kuczynski, J., Charlson, E. S., Zaneveld, J., Mozer, M. C., Collman, R. G., Bushman, F. D., Knight, R., & Kelley, S. T. (2011). Bayesian community-wide culture-independent microbial source tracking. *Nature Methods, 8*(9), 761–763. https://doi.org/10.1038/nmeth.1650.

28. Krawczyk, P., Lipinski, L., & Dziembowski, A. (2018). PlasFlow: predicting plasmid sequences in metagenomic data using genome signatures. *Nucleic Acids Research, 46* (6), e35. https://doi.org/10.1093/nar/gkx1321.

29. Langmead, B., & Salzberg, S. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods, 9*(4), 357–359. https://doi.org/10.1038/nmeth.1923.

30. Leitão, J. (2020). Microbial virulence factors. *International Journal of Molecular Sciences, 21*(15), 5320. https://doi.org/10.3390/ijms21155320.

31. Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Research, 49*(W1), W293–W296. https://doi.org/10.1093/nar/gkab301.

32. Li, D., Luo, R., Liu, C.-M., Leung, C.-M., Ting, H.-F., Sadakane, K., Yamashita, H., & Lam, T.-W. (2016). MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods, 102*, 3–11. https://doi.org/10.1016/j.ymeth.2016.02.020.

33. Liu, R. (2013). Health-promoting components of fruits and vegetables in the diet. *Advances in Nutrition, 4*(3), 384S–392S. https://doi.org/10.3945/an.112.003517.

34. Maggio, F., Rossi, C., Chavez-Lopez, C., Serio, A., Valbonetti, L., Pomilio, F., Chiavaroli, A. P., & Paparella, A. (2021). Interactions between *L. monocytogenes* and *P. fluorescens* in dual-species biofilms under simulated dairy processing conditions. *Foods, 10*(1), 176. doi: 10.3390/foods10010176.

35. Mann, E., & Wozniak, D. (2012). *Pseudomonas* biofilm matrix composition and niche biology. *FEMS Microbiology Reviews, 36*(4), 893–916. https://doi.org/10.1111/j.1574-6976.2011.00322.x.

36. Mhete, M., Eze, P., Rahube, T., & Akinyemi, F. (2020). Soil properties influence bacterial abundance and diversity under different land-use regimes in semi-arid environments. *Scientific African, 7*, e00246. https://doi.org/10.1016/j.sciaf.2019.e00246.

37. Møretrø, T., & Langsrud, S. (2017). Residential bacteria on surfaces in the food industry and their implications for food safety and quality. *Comprehensive Reviews in Food Science and Food Safety, 16*(5), 1022–1041. https://doi.org/10.1111/1541- 4337.12283.

38. Nasr, A., Mostafa, M., Arnaout, H., & Elshimy, A. (2018). The effect of exposure to sub-inhibitory concentrations of hypochlorite and quaternary ammonium compounds on antimicrobial susceptibility of *Pseudomonas aeruginosa*. *American Journal of Infection Control, 46*(7), e57–e63. https://doi.org/10.1016/j.ajic.2018.04.201.

39. Ondov, B. D., Treangen, T. J., Melsted, P., Mallonee, A. B., Bergman, N. H., Koren, S., & Phillippy, A. (2016). Mash: Fast genome and metagenome distance estimation using MinHash. *Genome Biology, 17*(1), 132. https://doi.org/10.1186/s13059-016-0997-x.

40. Oniciuc, E.-A., Likotra ti, E., Alvarez-Molina, A., Pietro, M., Lopez, M., & Alvarez-Ordóñez, A. (2019). Food processing as a risk factor for antimicrobial resistance spread along the food chain. *Current Opinion in Food Science, 30*, 21–26. https://doi.org/10.1016/j.cofs.2018.09.002.

41. Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., & Tyson, G. W. (2015). CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research, 25*(7), 1043–1055. https://doi.org/10.1101/gr.186072.114.

42. Pasolli, E., Asnicar, F., Manara, S., Zolfo, M., Karcher, N., Armanini, F., Beghini, F., Manghi, P., Tett, A., Ghensi, P., Collado, M. C., Rice, B. L., DuLong, C., Morgan, X. C.,

Golden, C. D., Quince, C., Huttenhower, C., & Segata, N. (2019). Extensive unexplored human microbiome diversity revealed by over 150,000 genomes from metagenomes spanning age, geography, and lifestyle. *Cell, 176*(3), 649–662. https://doi.org/10.1016/j.cell.2019.01.001.

43. Pericolini, E., Colombari, B., Ferretti, G., Iseppi, R., Ardizzoni, A., Girardis, M., Sala, A., Peppoloni, S., & Blasi, E. (2018). Real-time monitoring of *Pseudomonas aeruginosa* biofilm formation on endotracheal tubes in vitro. *BMC Microbiology, 18*(1), 84. https://doi.org/10.1186/s12866-018-1224-6.
44. Piovesan Pereira, B., Wang, X., & Tagkopoulos, I. (2021). Biocide-induced emergence of antibiotic resistance in *Escherichia coli*. *Frontiers in Microbiology, 12*, Article 640923. https://doi.org/10.3389/fmicb.2021.640923.
45. Puga, C. H., Dahdouh, E., SanJose, C., & Orgaz, B. (2018). *Listeria monocytogenes* colonizes *Pseudomonas fluorescens* biofilms and induces matrix over-production. *Frontiers in Microbiology, 9*, 1706. https://doi.org/10.3389/fmicb.2018.01706.
46. Quijada, N., Rodríguez-Lázaro, D., Eiros, J., & Hernández, M. (2019). TORMES: An automated pipeline for whole bacterial genome analysis. *Bioinformatics, 35*(21), 4207–4212. https://doi.org/10.1093/bioinformatics/btz220.
47. Quintieri, L., Caputo, L., Brasca, M., & Fanelli, F. (2021). Recent Advances in the mechanisms and regulation of QS in dairy spoilage by *Pseudomonas*. *Foods, 10*(12), 3088. https://doi.org/10.3390/foods10123088.
48. Randhawa, M. A., Khan, A. A., Javes, M. S. & Sajid, M. W. (2015). Chapter 18 - green leafy vegetables: A health promoting source. In R. R. Watson (Ed.), *Handbook of fertility, nutrition, diet, lifestyle and reproductive health* (pp. 205–220). Academic Press.
49. Rosenquist, H., Smidt, L., Andersen, S. R., Jensen, G. B., & Wilcks, A. (2005). Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. *FEMS Microbiology Letters, 250*(1), 129–136. https://doi.org/10.1016/j.femsle.2005.06.054.

50. Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics, 27*(6), 863–864. https://doi.org/10.1093/bioinformatics/btr026.

51. Schwengers, O., Barth, P., Falgenhauer, L., Hain, T., Chakraborty, T., & Goesmann, A. (2020). Platon: Identification and characterization of bacterial plasmid contigs in short-read draft assemblies exploiting protein sequence-based replicon distribution scores. *Microbial Genomics, 6*(10), mgen000398. https://doi.org/10.1099/mgen.0.000398.

52. Seemann, T. (2014). Prokka: Rapid prokaryotic genome annotation. *Bioinformatics, 30* (14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.

53. Senesi, S., & Ghelardi, E. (2010). Production, secretion and biological activity of *Bacillus cereus* enterotoxins. *Toxins (Basel), 2*(7), 1690–1703. https://doi.org/10.3390/toxins2071690.

54. Sequino, G., Valentino, V., Villani, F., & De Filippis, F. (2022). Omics-based monitoring of microbial dynamics across the food chain for the improvement of food safety and quality. *Food Research Internationa, 157*, Article 111242. https://doi.org/10.1016/j.foodres.2022.111242

55. Simonin, M., Briand, M., Chesneau, G., Rochefort, A., Marais, C., Sarniguet, A., & Barret, M. (2022). Seed microbiota revealed by a large-scale meta-analysis including 50 plant species. *New Phytologist, 234*(4), 1448–1463. https://doi.org/10.1111/nph.18037.

56. Soto-Giron, M. J., Kim, J.-N., Schott, E., Tahmin, C., Ishoey, T., Mincer, T. J., DeWalt, J., & Toledo, G. (2021). The Edible Plant Microbiome represents a diverse genetic reservoir with functional potential in the human host. *Scienti c Reports, 11*(1), 24017. https://doi.org/10.1038/s41598-021-03334-4.

57. Stellato, G., La Storia, A., De Filippis, F., Borriello, G., Villani, F., & Ercolini, D. (2016). Overlap of spoilage-associated microbiota between meat and the meat processing environment in small-scale and large-scale retail distributions. *Applied and Environmental Microbiology, 82*(13), 4045–4054. https://doi.org/10.1128/AEM.00793-16.

58. Taffner, J., Laggner, O., Wolfgang, A., Coyne, D., & Berg, G. (2020). Exploring the microbiota of East African indigenous leafy greens for plant growth, health, and resilience. *Frontiers in Microbiology, 11*, Article 585690. https://doi.org/10.3389/fmicb.2020.585690.

59. Tarricone, R., Rognoni, C., Arnoldo, L., Mazzacane, S., & Caselli, E. (2020). A probiotic-based sanitation system for the reduction of healthcare associated infections and antimicrobial resistances: A budget impact analysis. *Pathogens, 9*(6), 502. https://doi.org/10.3390/pathogens9060502.

60. Tatsika, S., Karamanoli, K., Karayanni, H., & Genitsaris, S. (2019). Metagenomic characterization of bacterial communities on ready-to-eat vegetables and effects of household washing on their diversity and composition. *Pathogens, 8*(1), 37. https:// doi.org/10.3390/pathogens8010037

61. Ventola, C. L. (2015). The antibiotic resistance crisis. *P&T, 40*(4), 277–283.

62. Wales, A., & Davies, R. (2015). Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. *Antibiotics (Basel), 4*(4), 567–604. https://doi.org/10.3390/antibiotics4040567.

63. Walterson, A., & Stavrinides, J. (2015). *Pantoea*: Insights into a highly versatile and diverse genus within the Enterobacteriaceae. *FEMS Microbiology Reviews, 39*(6), 968–984. https://doi.org/10.1093/femsre/fuv027.

64. Wang, F., Sun, R., Hu, H., Duan, G., Meng, L., & Qiao, M. (2022). The overlap of soil and vegetable microbes drives the transfer of antibiotic resistance genes from manure-amended soil to vegetables. *Science of the Total Environment, 828*, Article 154463. https://doi.org/10.1016/j.scitotenv.2022.154463.

65. Wood, D., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology,* 20(1), 257. https://doi.org/10.1186/s13059-019-1891-0.

66. World Health Organization (2018). *Critically important antimicrobials for human medicine, 6th revision*. Retrieved from https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf. Accessed June 1, 2022.

67. World Health Organization (2020). *Healthy diet*. Retrieved from https://www.who.int/news-room/fact-sheets/detail/healthy-diet/. Accessed June 1, 2022.

68. Xu, J.-G., Meng, J., Bao, W.-J., Kang, J.-M., Chen, J.-Y., & Han, B.-Z. (2021). Occurrence of disinfectant-resistant bacteria in a fresh-cut vegetables processing facility and their role in protecting *Salmonella enteritidis*. *RSC Advances, 11*(17), 10291–10299. https://doi.org/10.1039/d0ra09325d.

69. Yannarell, S. M., Grandchamp, G. M., Chen, S.-Y., Daniels, K. E., & Shank, E. A. (2019). A Dual-species biofilm with emergent mechanical and protective properties. *Journal of Bacteriology, 201*(18), e00670–e00718. https://doi.org/10.1128/JB.00670-18.

70. Yin, Y., Zhu, D., Yang, G., Su, J., & Duan, G. (2022). Diverse antibiotic resistance genes and potential pathogens inhabit in the phyllosphere of fresh vegetables. *Science of the Total Environment, 815*, Article 152851. https://doi.org/10.1016/j.scitotenv.2021.152851.

71. Yu, P., Yu, S., Wang, J., Guo, H., Zhang, Y., Liao, X., Zhang, J., Wu, S., Gu, Q., Xue, L., Zeng, H., Pang, R., Lei, T. Zhang, J., Wu, Q., & Ding, Y. (2019). *Bacillus cereus* isolated from vegetables in China: Incidence, genetic diversity, virulence genes, and antimicrobial resistance. *Frontiers in Microbiology,* 10, 948. https://doi.org/10.3389/fmicb.2019.00948.

## 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.

***References***

1. European Centre for Disease Prevention and Control. (2022). Assessing the health burden of infections with antibiotic-resistant bacteria in the EU/EEA, 2016-2020. Retrieved from https://www.ecdc.europa.eu/sites/default/files/documents/Health-burden-infections-antibiotic-resistant-bacteria.pdf. Accessed January 10, 2023.

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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.
2. **Valentino, V.**, Sequino, G., Cobo-Díaz, J. F., Alvarez-Ordóñez, A., De Filippis, F., & Ercolini, D. (2022). Evidence of virulence and antibiotic resistance genes from the microbiome mapping in minimally processed vegetables producing facilities. *Food Research International, 162,* article 112202. https://doi.org/10.1016/j.foodres.2022.112202.

***Publications not included in the thesis***

1. **Valentino, V.**, De Filippis, F., Menghi, L., Gasperi, F., & Ercolini, D. (2022). Food Neophobia and scarce olfactory performances are linked to oral microbiota. *Food Research International, 155,* article 111092. https://doi.org/10.1016/j.foodres.2022.111092.
2. Sequino, G., **Valentino, V.**, Villani, F., & De Filippis, F. (2022). Omics-based monitoring of microbial dynamics across the food chain for the improvement of food safety and quality. *Food Research International, 157,* article 111242. https://doi.org/10.1016/j.foodres.2022.111242.
3. De Franchis, R., Bozza, L., Canale, P., Chiacchio, M., Cortese, P., D'Avino, A., De Giovanni, M., Dello Iacovo, M., D'Onofrio, A., Federico, A., Gasparini, N., Iaccarino, F., Romano, G., Spadaro, R., Tedesco, M., Vitiello, G., Antignani, A., Auricchio, S., **Valentino, V.**, De Filippis, F., Ercolini, D., & Bruzzese, D. (2022). The Effect of Weaning with Adult Food Typical of the Mediterranean Diet on Taste Development and Eating

Habits of Children: A Randomized Trial. *Nutrients, 14,* 2486. https://doi.org/10.3390/nu14122486.

4. Carucci, L., Nocerino, R., Paparo, L., De Filippis, F., Giglio, V., Cozzolino, T., **Valentino, V.**, Sequino, G., Bedogni, G., Russo, R., Ercolini, D., & Berni Canani, R. (2022). Therapeutic effects elicited by the probiotic Lacticaseibacillus rhamnosus GG in children with atopic dermatitis. The results of the ProPAD trial. *Pediatric Allergy and Immunology, 33,* e13836. https://doi.org/10.1111/pai.13836.
5. Sequino, G.*, **Valentino, V.***, Torrieri, E., & De Filippis, F. (2022). Specific Microbial Communities Are Selected in Minimally-Processed Fruit and Vegetables according to the Type of Product. *Foods, 11,* 2164. https://doi.org/10.3390/ foods11142164.