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Environmental microbiome mapping as a strategy to improve quality and safety in the food industry

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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. These complex microbial consortia may include microbial spoilers, pathogens, as well as beneficial microbes.

Advances in sequencing technologies and metagenomics provide the opportunity to map the environmental microbiome in food industries at an unprecedented depth, highlighting the importance of the resident microbial communities in influencing food quality and safety, as well as the main factors shaping its composition and activities. However, specific technical issues must be considered. Although microbiome mapping in the food industry has the potential to revolutionize food safety and quality management systems, its application as routine practice is still challenging and technical issues limit the exploitation of the powerful information that can be obtained by the application of such state-of-the-art approaches.

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Food processing facilities are inhabited by a resident microbiome

Microbial contamination in food processing environments 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, surfaces that are not in direct contact with foods 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 [1]. 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.

In the last decade, metagenomics has begun popular for microbiome mapping in food handling or processing facilities (Table 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 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 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 [18,19,20,23], ready-to-eat, composite meals [21] and fresh fruit [15]. 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, for example, temperature, gaseous atmosphere employed. Moreover, some studies also report

Table 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	[2]
Bakery	4	*Saccharomyces cerevisiae, Weissella, Lactobacillus, Leuconostoc, Bacillus, Streptococcus, Pseudomonas, Staphylococcus*	Yes	Dough mixer, storage boxes, walls	*Staphylococcus*	Yes	[3]
Brewery	1	*Saccharomyces cerevisiae, Kocuria, Micrococcus, Acinetobacter, Pediococcus*	Yes	Fermentation tanks, drain, sink, barrels	No	Yes	[4]
Cheeses	1	*Leuconostoc citreum, Pseudomonas, Lactococcus lactis*	NA	Floor drains	*Listeria monocytogenes*	Yes	[5]
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	[6]
Cheeses, pasta-filata	1	*Macrococcus caseolyticus, Lactococcus lactis*	Yes	Curd vat, draining table, knives, brining tank, stretching and molding machines	No	Yes	[7]
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	[8••]
Cheeses, smear-ripened	1	*Lactobacillus kefiranofaciens, Streptococcus thermophilus, Debaryomyces hansenii, Saccharomyces unisporus*	Yes	Floor drains	No	Yes	[9]
Cheeses, smear-ripened	2	*Debariomyces, Lactococcus, Staphylococcus, Brevibacterium*	Yes	Drains, aging racks, tanks, draining table	*Staphylococcus*	Yes	[10]
Cheeses, smear-ripened	1	*Streptococcus, Staphylococcus, Lactococcus, Pseudomonas*	Yes	Cow teats, milk tanks, molds, packaging, aging shelves	No	Yes	[11]
Cheeses, smear-ripened	1	*Brevibacterium, Corynebacterium, Debariomyces, Galactomyces*	Yes	Wooden aging shelves	No	Yes	[12]
Cheeses, washed rinds	2	*Halomonas, Corynebacterium, Staphylococcus, Brevibacterium*	Yes	Aging shelves and racks, walls, floors	*Staphylococcus*	Yes	[13]
Chinese liquor	1	*Lactobacillus, Leuconostoc, Pseudomonas, Saccharomyces, Rhizopus, Rhizomucur*	Yes	Fermentation jar	*Staphylococcus*	Yes	[14]
Fruit packing	3	*Pseudomonadaceae, Flavobacteriaceae, Xanthomonadaceae, Aureobasidiaceae, Aspergillaceae*	Yes	Floors	*Listeria monocytogenes*	No	[15]
Milk	1	*Lactococcus, Acinetobacter, Streptococcus, Staphylococcus, Bacillus*	Yes	Silos, pasteurizers, concentrators	*Staphylococcus*	Yes	[16]
Milk		*Streptococcus, Pseudomonas, Staphylococcus, Enterobacteriaceae*	Yes	Tanker tucks	*Staphylococcus*	Yes	[17]
Raw meat, sausages	1	*Brochothrix, Leuconostoc, Lactobacillus, Yersinia*	Yes	Transport belt, meat emulsion blender, filling machine, trolleys	*Yersinia*	No	[18]
Raw meat, steaks	20	*Brochothrix, Pseudomonas, Psychrobacter, Streptococcus*	Yes	Chopping boards, knives, operator hands	No	No	[19]

Table 1 (*Continued*)

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
Raw meat, steaks	1	*Brochothrix, Pseudomonas, Psychrobacter, Streptococcus*	Yes	Chopping boards, knives, operator hands, cold-store walls, beef carcass	No	No	[20]
Ready-to-eat meals	2	*Leuconostoc, Lactobacillus, Streptococcaceae, Pseudomonas*	Yes	Mixing vessel, bench, carrier vessel, mixing machine, washing tank, dicer	No	No	[21]
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	[22]
Salmon fillets	2	*Pseudomonas, Shewanella*	Yes	Seawater tanks, conveyors, gutting machine	No	No	[23]
Winery	1	*Saccharomyces cerevisiae, Hanseniaspora uvarum, Brevundimonas, Comamonadaceae, Enterobacteriaceae*	Yes	Grape crusher, press, fermentor, pump, barrels, drain	No	Yes	[24]

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 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 1). This was highlighted in several studies involving fermented dairy products or beverages (Table 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 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 [25,26]. 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.

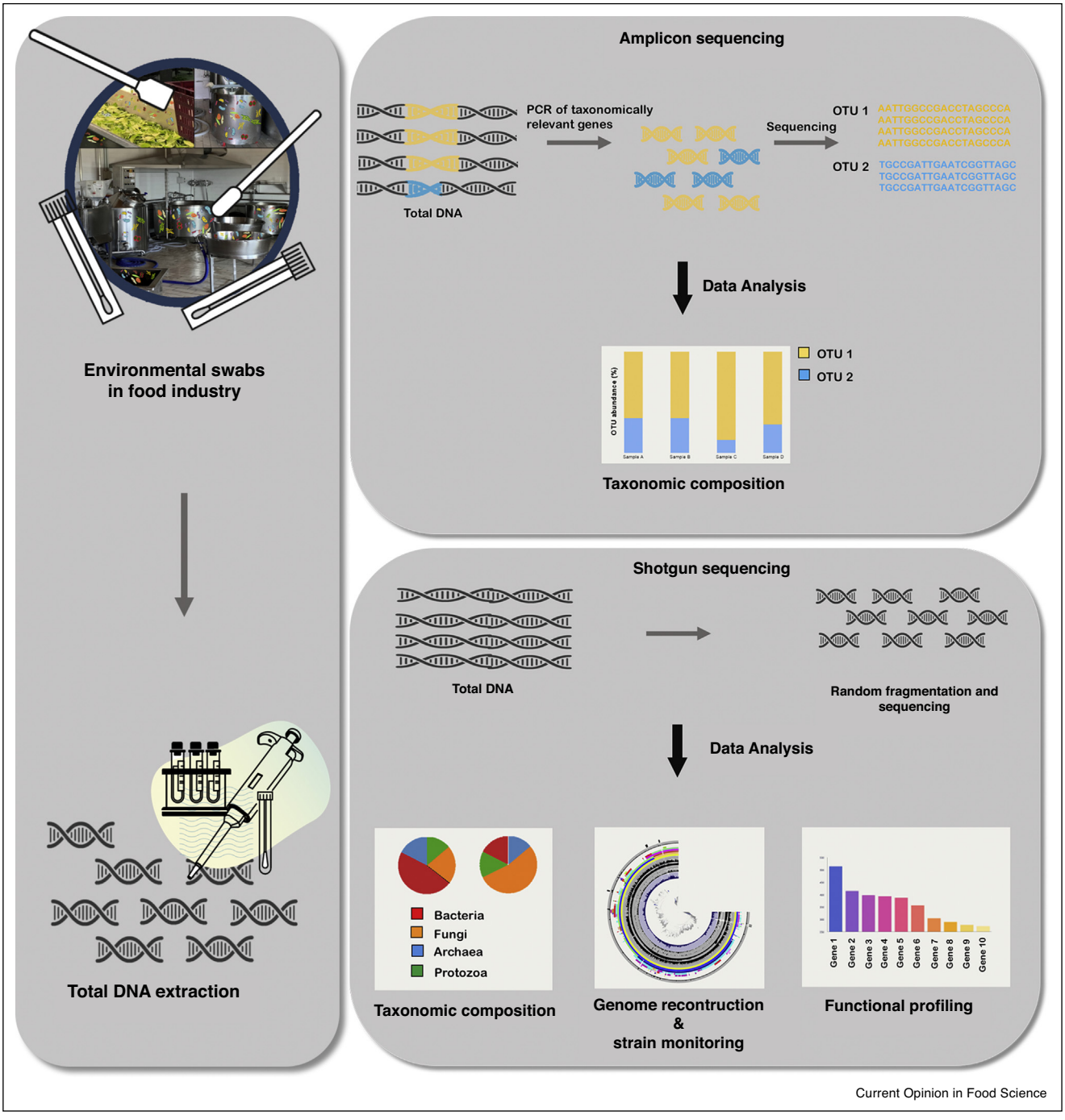
Metagenomics-based microbiome mapping in food processing environments

Microbial colonization of surfaces and tools in the food processing environments is a widespread phenomenon [27•], 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 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 1). Depending on their composition and hygienic conditions, ingredients, raw materials and processing water entering the food

Figure 1

Factors influencing environmental microbiome in food industry.

Figure 2



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

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 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 disinfection practices, temperature shifts (e.g. during different seasons) and other factors (Figure 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). When preparing for HTS-based profiling, amplicon-based or shotgun-based approaches can be considered. For the former, a gene of taxonomic relevance, for example, 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). 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 [28,29••]. 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). 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). 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 1; [30•]). This approach, which is easier and cheaper but less informative than SM, may be introduced to support routine quality and safety management plans. 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 the resident microbiome in a food facility is possible [4,8••]. 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). 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.

Limitations and technical warnings

DNA sequencing and library preparation costs have dramatically decreased over time [31•], making both amplicon-sequencing and shotgun-sequencing more affordable, as demonstrated by the increasing number of studies in which these techniques are used. However, the actual sequencing cost of a pool of environmental samples may vary significantly, depending on the sequencing platform, on the approach used (e.g. shotgun-based or amplicon-based) [32] and on the required sequencing depth [33]. Therefore, the sampling scheme and the type of approach to be used should be carefully evaluated according to the industry requirements: cheaper amplicon-based microbiome mapping can be used for a routine control, while more expensive shotgun sequencing approaches can be useful when a deeper level of information is needed.

Moreover, 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, that is, below 2.5 CFU/cm^2 in most cases [1], 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 [34]. Although this provides a means of increasing workable DNA amounts, it is well-documented that this approach may represent a source of bias [35]. Indeed, when comparing two popular MDA kits, Yilmaz *et al.* [36] 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). 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 [37]. 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). 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 [38•,39], while polyurethane swabs are well-suited for sampling porous surfaces [37]. However, experimental data indicate that microbial adhesion strongly depends on the features of the surface being sampled [40] and on factors such as the presence of exopolysaccharides and the frequency and intensity of cleaning procedures [41]. Moreover, Motz *et al.* [42] 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.

Table 2

Different types of swabs available on the market

Swab types	Advantages	Disadvantages	References
Cotton and rayon swabs	Low cost Most used	May release of impurities May entrap bacterial cells Plant DNA contamination	[34]
Swabs made of foam (e.g. polyurethane)	Suitable for porous surfaces	Hydrophobicity may limit buffer absorption	[34] [35]
Nylon flocked swabs	High capillarity improves cell release	Swab material may be released on rough surfaces	[36] [37]

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 [43]. 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. The most common steps in metagenomic data analysis consist of quality filtering of the reads, taxonomic and/or functional profiling of metagenomes and the reconstruction of metagenomes-assembled genomes (MAGs). For each of the metagenomic data analysis steps, several tools and analysis pipelines are available, and a deep knowledge of algorithms and tool-specific settings is needed. In addition, to get meaningful results, data analysis must be followed by a statistical exploration and results summarized in tables or plots. 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.

Microbiome mapping and EU/US 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. Such a preventive approach has been adopted in the US too, with the publication of the FDA Food Safety Modernization Act in 2011. Since then, all the facilities under FDA jurisdiction (with some exceptions) have been required to adopt the Hazard Analysis and Risk-based Preventive Controls (HARPC) principles.

In the EU, 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. In the US, the Code of Federal Regulations clearly states that, if the food business operator considers contamination of ready-to-eat food a hazard requiring preventive controls, environmental monitoring must be carried out to verify their effectiveness. 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 [44]. 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.

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.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Francesca De Filippis: Conceptualization, Formal analysis, Writing - original draft. **Vincenzo Valentino:** Formal analysis, Writing - original draft. **Avelino Alvarez-Ordóñez:** Formal analysis, Writing - original draft, Funding acquisition. **Paul D Cotter:** Resources, Writing - review & editing, Funding acquisition. **Danilo Ercolini:** Conceptualization, Resources, Writing - review & editing, Funding acquisition.

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- • of special interest
- •• of outstanding interest

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