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Recent Past, Present, and Future of the Food Microbiome

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Keywords

amplicon sequencing, food microbiome, food microbiota, high-throughput sequencing, metagenomics, metatranscriptomics, whole-genome sequencing

Abstract

Sequencing technologies have deeply changed our approach to the study of food microbial communities. This review describes recent exploitations of high-throughput sequencing applications to improve our knowledge of food microbial consortia. In the past 10 years, target amplicon sequencing has become routinely used in many food microbiology laboratories, providing a detailed picture of food-associated microbiota. Metagenomics and metatranscriptomics approaches are still underexploited in food microbial ecology, despite their potential to uncover the functionality of complex communities. In a near future, sequencing technologies will surely advance our understanding of how to effectively use the invaluable microbial resources to improve food quality and safety.

INTRODUCTION

The relationship between foods and their microbiome is fundamental to their quality and safety. Beneficial microbial communities can be responsible for rheological and organoleptic traits of fermented foods. However, undesirable microbes may also be present, and their development may affect the quality of food, leading to spoilage or other food safety issues. Food microbiology has traditionally relied on culture-based techniques. However, in addition to their low sensitivity, such techniques may require unknown growth factors and/or growth conditions present in natural habitats but not easy to reproduce in laboratory media. Such limitations may lead to an underestimation of microbial diversity. Culture-independent techniques, based on the analysis of nucleic acids (NAs) extracted directly from the food matrix, can help in overcoming these limitations. Different methods have been widely applied in microbial ecology studies, with denaturing gradient gel electrophoresis, temporal temperature gradient gel electrophoresis, and real-time quantitative polymerase chain reaction (qPCR) the most common techniques used in food microbial ecology (Cocolin et al. 2013).

The advent of high-throughput sequencing (HTS) technologies in 2004 revolutionized our approach to microbial ecology. After the launch on the market of the first pyrosequencer by 454 Life Sciences in 2004 and of Genome Analyzer by Solexa in 2005, different sequencers followed, with constant improvements in throughput (number of reads produced per single run) and read length (Mayo et al. 2014). HTS ensures higher sensitivity compared with traditional culture-independent approaches, allowing the detection of nondominant communities that may play an important role in the studied ecosystem. However, the great advantage of these methods is the unprecedented potential for quantitative detection of the structure of microbial communities: The number of reads detected for a given organism is proportional to its abundance in the sample. Moreover, HTS greatly reduced the price per base compared to Sanger sequencing, boosting the spread of these technologies in microbiology laboratories and their application in multiple fields. A search of the Web of Science database (**https://apps.webofknowledge.com**) for "food" AND either "microbiota" OR "microbiome" produced 17,916 hits published from 2008 to (April) 2017, with a huge increase in the number of published papers beginning in 2011–2012, when HTS technologies started to be routinely used in food microbial ecology studies (**Figure 1**). Two

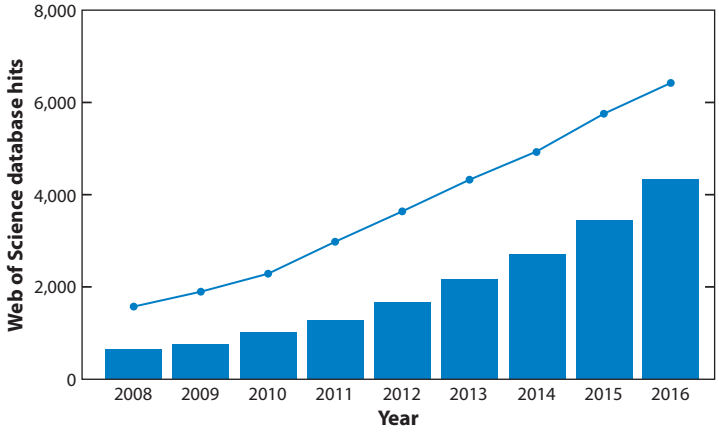


Figure 1

Number of hits found in Web of Science (**https://apps.webofknowledge.com**) database for search terms "food microbiome" or "food microbiota" (*bar graph*), and "high-throughput sequencing" (*line graph*) in articles published from 2008 to 2016 (search on April 2017).

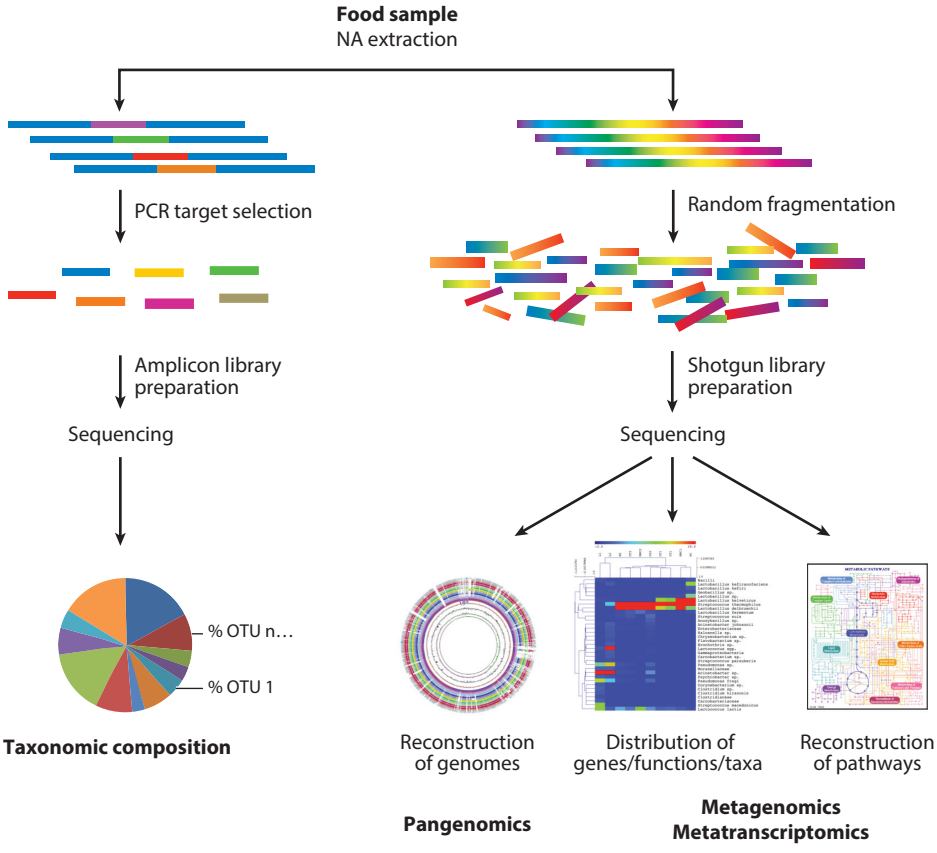


Figure 2

Graphic representation of possible high-throughput sequencing applications for the study of food microbiota or microbiome. Abbreviations: NA, nucleic acid; OTU, operational taxonomic unit; PCR, polymerase chain reaction.

substantially different approaches can be used: targeted amplicon sequencing and shotgun sequencing (**Figure 2**). In both cases, NAs are directly extracted from food samples. When the amplicon-based approach is employed, a PCR step is necessary for the selection of the desired target gene. Taxonomically relevant genes are the common targets of this analysis, with the 16S ribosomal RNA (rRNA) gene considered the universal target for bacteria. Reads obtained can be aligned to appropriate databases, thereby obtaining the taxonomic composition of the microbiota in a given sample. Besides the identification of the operational taxonomic units (OTUs) present, their relative abundance can be estimated, because the number of reads associated with a given taxon will be proportional to its levels in the sample. In the shotgun-based approach, the whole DNA or RNA (after the synthesis of complementary DNA) is fragmented by enzymatic or mechanical methods prior to being sequenced. The final output represents the whole genomic potential (metagenome) of the microbial populations present in the sample: Bioinformatics analysis allows identification of the presence and abundance of specific genes of interest and reconstruction of metabolic pathways in addition to providing information on taxonomic composition. When RNA, in particular, messenger RNA (mRNA), is the target of this analysis, a picture of the metatranscriptome can be achieved.

Metagenome: all the microbial genomes present in an environmental sample; indicative of the potential activities of the microbial community

Metatranscriptome: the pool of genes actually expressed in an environmental sample

Shotgun-based DNA sequencing may also be used when starting from the DNA of a pure culture as a quick and cost-effective method for whole-genome sequencing (WGS). Thanks to the reduced sequencing costs, several complete and draft genomes of different strains of the same microbial species are now available in public databases. This allows comparative genomics studies to identify the core genome and pangenome of a species. One promising application offers the potential to reconstruct draft and complete genomes directly from metagenomics reads, avoiding previous cultivation and isolation, overcoming the limitation of the culture-based techniques, and allowing in situ strain monitoring.

Pangenome: the entire set of genes found in a microbial species, including those genes present in all strains (core genome) and those present in only some

Below, the application of metagenomics in food microbial ecology studies is described, with emphasis on the use of metagenomics in the monitoring of food fermentation or spoilage dynamics and in food safety. Finally, recent promising approaches for data analysis are described and issues and pitfalls are discussed.

FOOD MICROBIAL ECOLOGY STUDIES

High-Throughput Investigation of Food Fermentations

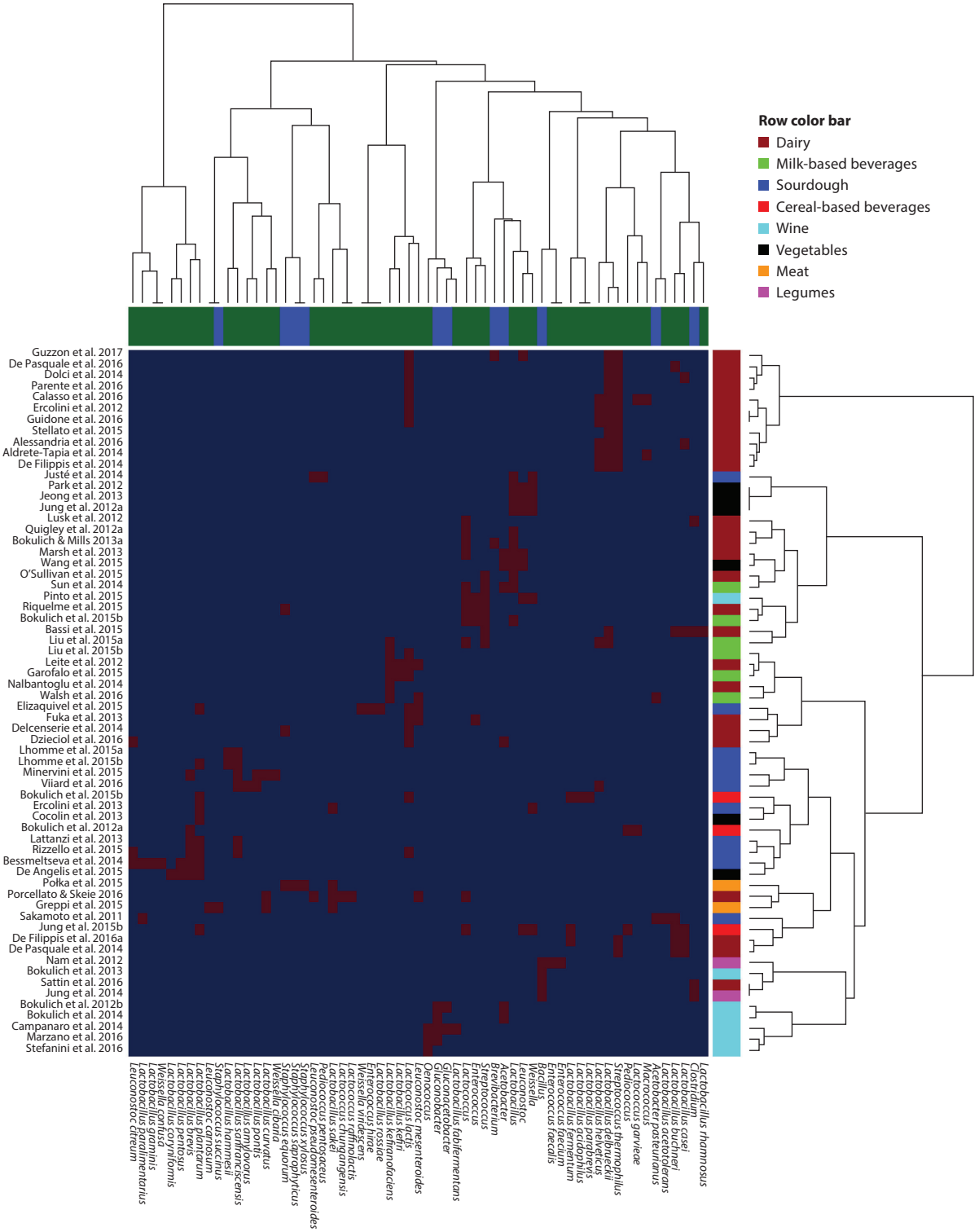
Thus far, most of the HTS-based studies discussed have focused on the monitoring of microbial populations during food fermentations (De Filippis et al. 2017c). A comprehensive, although not exhaustive, list of studies using amplicon-based HTS in foods and food environments is provided in **Supplemental Table 1**. HTS has been useful in characterizing the microbiota involved in the manufacturing and ripening of several traditional fermented products (**Figure 3**). Most of the studies relied on the use of amplicon-targeted HTS, which gave a comprehensive and more sensitive picture of the microbiota but provided only a description of the populations involved and did not add appreciably to our knowledge of the microbiota of fermented foods. Lactic acid bacteria (LAB) were confirmed as the main players in many food fermentations (**Figure 3**), although the higher sensitivity of this method highlighted the presence of minor communities never reported previously (Quigley et al. 2012b). After an initial publication on Irish cheeses (Quigley et al. 2012b), the microbial ecology of a wide variety of fresh and ripened dairy products was investigated (**Supplemental Table 1**). A comprehensive characterization of the microbiota in traditional cheeses that are granted a specific/protected labeling may help in defining their typicality and tracing their origin (Aldrete-Tapia et al. 2014; De Filippis et al. 2014, 2016a; Dolci et al. 2014; Delcenserie et al. 2014; Ercolini et al. 2012; Fuka et al. 2013). Moreover, a better understanding of the manufacturing and ripening process may be achieved, emphasizing differences in microbial composition in different parts of the same cheese (Calasso et al. 2016; De Filippis et al. 2016a; De Pasquale et al. 2014, 2016; O'Sullivan et al. 2015).

Supplemental Material

Besides cheeses, other types of food fermentations have been investigated using a sequencing-based taxonomic approach (**Figure 3**; **Supplemental Table 1**). Fermentation of sourdough used to produce traditional bread and sweet leavened products has also been explored. Although different types of flours harbor complex and diverse microbiota (Ercolini et al. 2013), as soon as fermentation proceeds, a selected core microbiota including few taxa can be identified in all the

Figure 3

Heat plot showing presence (*red*) or absence (*blue*) of the most abundant microbial taxa in food samples analyzed in 64 published studies (*listed on the left*). Only taxa with abundances greater than 1% (as reported in the original publication) are included. The row bar is colored according to the type of food matrix, whereas the column bar shows taxa belonging to lactic acid bacteria (*green*) or others (*blue*).


Row color bar
Dairy
Milk-based beverages
Sourdough
Cereal-based beverages
Wine
Vegetables
Meat
Legumes
Guzzon et al. 2017
De Pasquale et al. 2016
Dolci et al. 2014
Parente et al. 2016
Calasso et al. 2016
Ercolini et al. 2012
Guidone et al. 2016
Stellato et al. 2015
Alessandria et al. 2016
Aldrete-Tapia et al. 2014
De Filippis et al. 2014
Justé et al. 2014
Park et al. 2012
Jeong et al. 2013
Jung et al. 2012a
Lusk et al. 2012
Quigley et al. 2012a
Bokulich & Mills 2013a
Marsh et al. 2013
Wang et al. 2015
O'Sullivan et al. 2015
Sun et al. 2014
Pinto et al. 2015
Riquelme et al. 2015
Bokulich et al. 2015b
Bassi et al. 2015
Liu et al. 2015a
Liu et al. 2015b
Leite et al. 2012
Garofalo et al. 2015
Nalbantoglu et al. 2014
Walsh et al. 2016
Elizaquivel et al. 2015
Fuka et al. 2013
Delcenserie et al. 2014
Dzieciol et al. 2016
Lhomme et al. 2015a
Lhomme et al. 2015b
Minervini et al. 2015
Viiard et al. 2016
Bokulich et al. 2015b
Ercolini et al. 2013
Cocolin et al. 2013
Bokulich et al. 2012a
Lattanzi et al. 2013
Rizzello et al. 2015
Bessmeltseva et al. 2014
De Angelis et al. 2015
Połka et al. 2015
Porcellato & Skeie 2016
Greppi et al. 2015
Sakamoto et al. 2011
Jung et al. 2015b
De Filippis et al. 2016a
De Pasquale et al. 2014
Nam et al. 2012
Bokulich et al. 2013
Sattin et al. 2016
Jung et al. 2014
Bokulich et al. 2012b
Bokulich et al. 2014
Campanaro et al. 2014
Marzano et al. 2016
Stefanini et al. 2016
Lactobacillus rhamnosus
Clostridium
Lactobacillus casei
Lactobacillus buchneri
Lactobacillus acetotolerans
Acetobacter pasteurianus
Macrococcus
Pediococcus garvieae
Lactococcus garvieae
Streptococcus thermophilus
Lactobacillus delbrueckii
Lactobacillus helveticus
Lactobacillus acidophilus
Lactobacillus fermentum
Enterococcus faecium
Enterococcus faecalis
Bacillus
Weissella
Leuconostoc
Lactobacillus
Acetobacter
Brevibacterium
Streptococcus
Lactococcus
Lactobacillus fabifermentans
Gluconobacter
Gluconacetobacter
Oenococcus
Leuconostoc mesenteroides
Lactococcus lactis
Lactobacillus kefiri
Lactobacillus kefranofaciens
Lactococcus raffinolactis
Enterococcus hirae
Weissella viridescens
Lactococcus chungangensis
Pediococcus pentosaceus
Leuconostoc pseudomesenteroides
Staphylococcus xylosus
Staphylococcus saprophyticus
Staphylococcus equorum
Weissella cibaria
Lactobacillus curvatus
Lactobacillus pontis
Lactobacillus sanfranciscensis
Lactobacillus hammesii
Staphylococcus succinus
Leuconostoc carnosum
Lactobacillus plantarum
Lactobacillus paraplantarum
Lactobacillus pentosus
Lactobacillus coryniformis
Weissella confusa
Lactobacillus graminis
Lactobacillus parafarraginis
Leuconostoc citreum

samples during sourdough propagation (Ercolini et al. 2013, Lattanzi et al. 2013, Lhomme et al. 2015) (**Figure 3**).

Supplemental Material

The presence of a resident microbiota in food processing facilities has also been frequently investigated (**Supplemental Table 1**), describing the occurrence of beneficial microbes that may be involved in the fermentative process of cheese (Bokulich & Mills 2013a, Calasso et al. 2016, Stellato et al. 2015), sourdough (Minervini et al. 2015), and fermented beverages (Bokulich et al. 2012a, 2012b, 2015a). However, the facility environment may also be a primary source of potential spoilers or pathogens (Bokulich et al. 2015b, Stellato et al. 2015).

Microbiome studies are very often completed with a certain amount of metadata, the more the better. A great potential of taxonomic studies is the option to correlate the abundance of microbial taxa with other continuous variables. Such analyses do not prove causal effects and the results should be considered carefully, as the correlative link is not always due to ecologically meaningful relationships. However, statistically relevant associations can be very useful in supporting a hypothesis on the role of certain microbial species in the food. Correlation analysis between the abundance of microbial taxa and chemical determinations may highlight the possible species responsible for the production of metabolites important for the properties of the final products (De Filippis et al. 2017b, De Pasquale et al. 2014, Lattanzi et al. 2013).

The use of shotgun metagenomics and metatranscriptomics may give more useful information about the microbial activities involved in food production. Although this approach is still underexploited, a few studies have emphasized its potential in understanding the cheese-ripening process (De Filippis et al. 2016a, Dugat-Bony et al. 2015, Lessard et al. 2014, Monnet et al. 2016, Wolfe et al. 2014).

Wolfe et al. (2014) used metagenomics to study the main microbial activities contributing to the flavor of surface-ripened cheeses. Different surface-ripened cheeses (bloomy, natural, and washed-rind cheeses) were analyzed and pathways related to sulfur and branched-chain amino acid degradation were found to be enriched in washed-rind cheeses, leading to compounds characterized by more pungent aromas. Moreover, new cold-adapted lipase and protease were identified that are possibly involved in cheese flavor production during refrigerated cheese aging and storage (Wolfe et al. 2014). Metagenomics may help in understanding potential activities of the microbial community. In contrast, the use of metatranscriptomics may sometimes be preferable to identify which genes are expressed in the food and to observe patterns of functional change over time or in response to a modulation of the technological parameters (e.g., temperature, relative humidity). In other studies, model surface-ripened cheeses were inoculated with bacterial and fungal strains and their transcriptomes studied over the ripening period, highlighting changes that occur in the expression of microbial genes and pathways related to carbohydrate fermentation, proteolysis, and amino acid catabolism, which lead to the production of flavor-active molecules (Dugat-Bony et al. 2015, Lessard et al. 2014). In addition, Monnet et al. (2016) studied the metatranscriptome of inoculated strains during ripening of a Reblochon-type cheese, clarifying the role of the strains in the different phases of ripening. The authors suggested that the yeast *Geotrichum candidum* was important in the first phase of the ripening process when the expression of genes associated with amino acid catabolism from this strain reached a maximum. As ripening proceeds, *Debaryomyces hansenii* takes over, underlining a different role for the inoculated starters in determining the flavor and texture of the final cheese.

To date, only one research study has focused on the metatranscriptome of a cheese produced with the addition of an undefined natural starter (De Filippis et al. 2016a). The natural fermentations are particularly interesting cases in which microbial activities involve a complex and undefined microbiota, usually with unpredictable results in terms of manufacturing and ripening quality and kinetics. In a ripened pasta filata cheese, the effect on microbial gene expression of a change in the

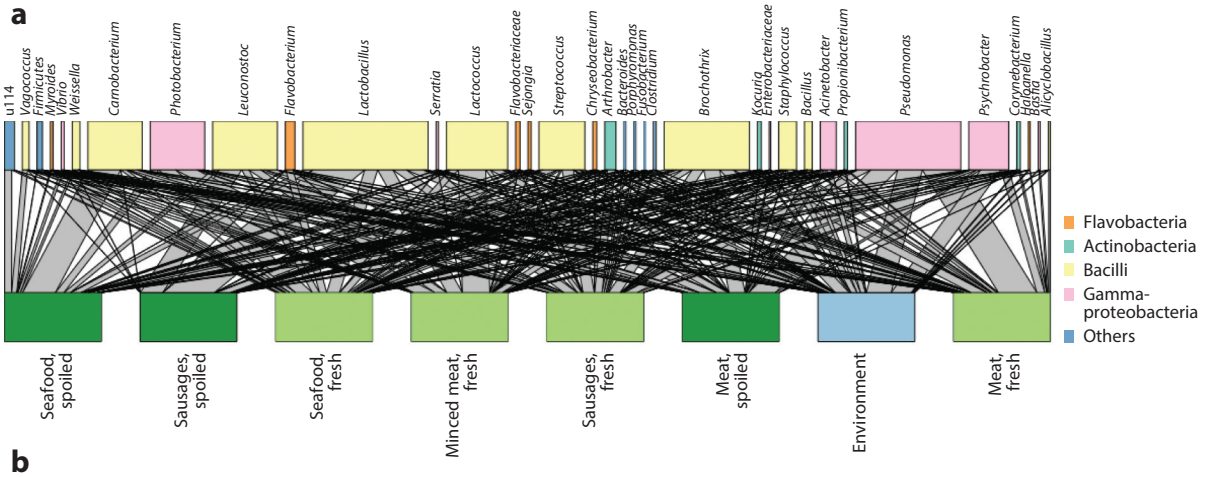
ripening parameters (humidity and temperature) was studied, showing how the increase of ripening temperature enhances the expression of genes involved in proteolysis, lipolysis, and amino acid and lipid catabolism in LAB, possibly influencing cheese texture and aroma (De Filippis et al. 2016a). Such information at the molecular level can be useful in predicting how technological parameters can be utilized to accelerate the dynamics of cheese ripening. Undefined starter cultures possess high genetic diversity, which may be important in flavor development. Erkus et al. (2013) used a combination of metagenomics and pangenomics to understand the mechanisms involved in maintaining the genetic diversity of an undefined milk starter used in cheese manufacturing during several propagation cycles. The authors suggested that a kill-the-winner mechanism subsists: The phage sensitivity of the fittest strain was regulated by its concentration in the culture, preventing the extinction of other genetic lineages during back-slopping routines (Erkus et al. 2013). Genomic comparison of foodborne bacteria and fungi may be very useful in understanding their adaptive mechanisms to the food matrix and highlighting the presence of technologically important differences among closely related strains (Ercolini 2017). Indeed, comparison of *Penicillium* spp. genomes enabled the identification of recent horizontally transferred elements present only in domesticated strains isolated from cheeses (Ropars et al. 2015). The authors identified genes on these elements that contributed to a competitive advantage in utilizing cheese nutrients, conferring to the strains carrying the genes the ability to grow faster in a cheese matrix (Ropars et al. 2015).

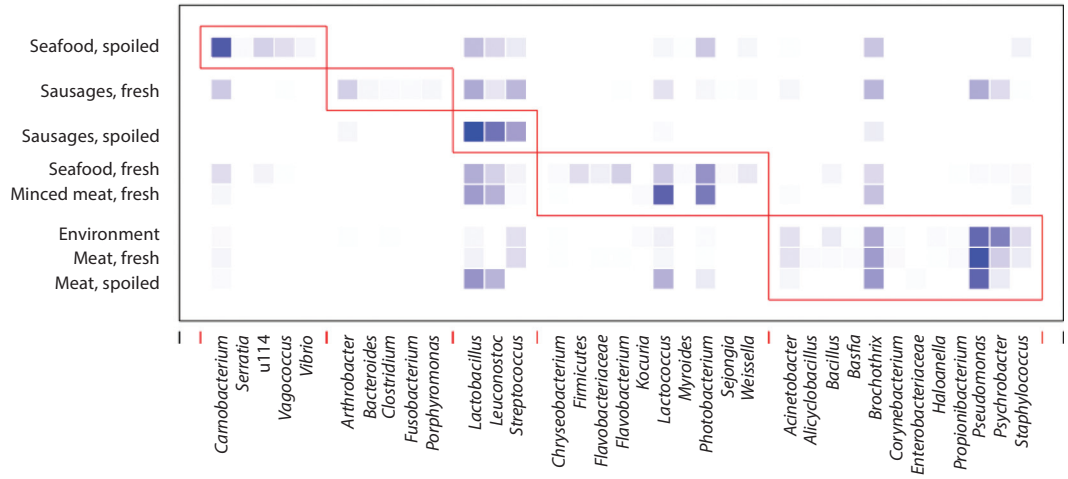
Only a few studies have addressed the potential of shotgun metagenomics in foods other than cheese, such as kimchi (Jung et al. 2013), Chinese rice wine (Hong et al. 2016), and cocoa beans (Illeghems et al. 2015). Nevertheless, omics-based approaches promise to reveal the contribution of fermentative microbes in several biotechnologically relevant processes. Application of omics will elucidate the pathways leading to the desired properties in different food products and help in understanding approaches to quality enhancement. Moreover, comparing genomes from food isolates, or those retrieved directly from metagenomics reads, has the potential to uncover the hidden properties of several foodborne microbes and thereby exploit them to enhance quality and safety.

Tracking Microbial Contamination Routes and Monitoring Food Spoilage

Understanding the evolution of the specific spoilage organisms (SSOs) in the dynamics of food spoilage is another key topic for food microbiologists. Most of the studies on this topic in the literature focus on fresh meat spoilage, monitoring the development of the SSOs during storage and/or their diversity changes according to different packaging conditions (**Supplemental Table 1**). **Figure 4** shows the most abundant microbial genera associated with the spoilage of seafood and several meat products (fresh meat, minced meat, raw sausages) in previously published studies extracted from FoodMicrobionet (**http://www.foodmicrobionet.org**) (Parente et al. 2016). FoodMicrobionet is a database that contains data from 33 studies on food-associated bacterial communities and related metadata. Samples are classified using the FoodEx classification (**http://www.efsa.europa.eu/en/data/data-standardisation**), and even inexperienced users can easily extract subsets of samples for the food matrix of interest and use them in comparative studies. Data can be easily processed for α- and β-diversity analyses using ad hoc R scripts (**https://www.r-project.org**). Fresh meat, fish, and environmental samples show high bacterial diversity that decreases during spoilage when SSOs are selected by the storage conditions used: The spoilage of sausages (stored in a vacuum) is characterized by the presence of LAB, whereas *Pseudomonas* and *Brochothrix* dominate in aerobically spoiled meat (**Figure 4*a*,*b***). Nevertheless, a spoilage-associated core microbiota can be observed, common to both meat and seafood products

Supplemental Material


a
u114
Vagococcus
Firmicutes
Myroides
Vibrio
Weissella
Carnobacterium
Photobacterium
Leuconostoc
Flavobacterium
Lactobacillus
Serratia
Lactococcus
Flavobacteriaceae
Sejongia
Streptococcus
Chryseobacterium
Arthrobacter
Bacteroides
Porphyromonas
Fusobacterium
Clostridium
Brochothrix
Kocuria
Enterobacteriaceae
Staphylococcus
Bacillus
Acinetobacter
Propionibacterium
Pseudomonas
Psychrobacter
Corynebacterium
Haloanella
Basfia
Alicyclobacillus
Flavobacteria
Actinobacteria
Bacilli
Gamma-proteobacteria
Others
Seafood, spoiled
Sausages, spoiled
Seafood, fresh
Minced meat, fresh
Sausages, fresh
Meat, spoiled
Environment
Meat, fresh


b
Seafood, spoiled
Sausages, fresh
Sausages, spoiled
Seafood, fresh
Minced meat, fresh
Environment
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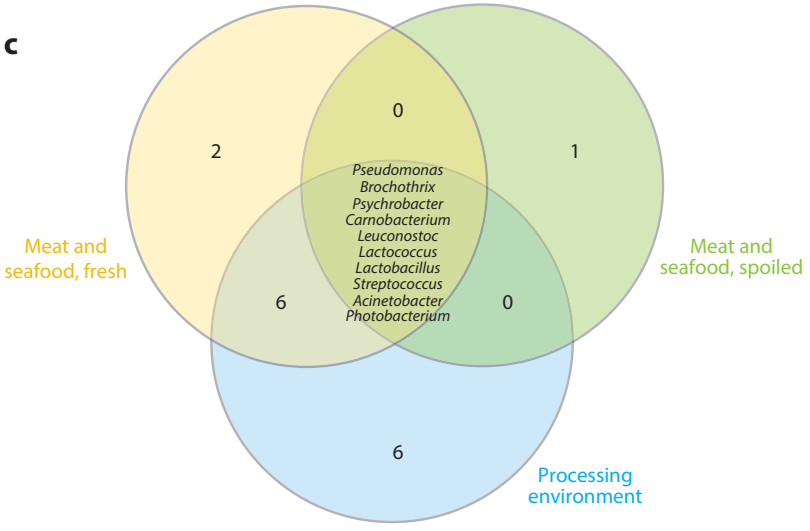
Meat, spoiled
Carnobacterium
Serratia
u114
Vagococcus
Vibrio
Arthrobacter
Bacteroides
Clostridium
Fusobacterium
Porphyromonas
Lactobacillus
Leuconostoc
Streptococcus
Chryseobacterium
Firmicutes
Flavobacteriaceae
Flavobacterium
Kocuria
Lactococcus
Myroides
Photobacterium
Sejongia
Weissella
Acinetobacter
Alicyclobacillus
Bacillus
Basfia
Brochothrix
Corynebacterium
Enterobacteriaceae
Haloanella
Propionibacterium
Pseudomonas
Psychrobacter
Staphylococcus


c
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1
Pseudomonas
Brochothrix
Psychrobacter
Carnobacterium
Leuconostoc
Lactococcus
Lactobacillus
Streptococcus
Acinetobacter
Photobacterium
Meat and seafood, fresh
Meat and seafood, spoiled
6
0
6
Processing environment

as well as to the production environment (**Figure 4*c***), highlighting the importance of the food processing environment as a source of potential spoilage microbes. These types of observations may help to identify the main microbial players in the spoilage process along with their potential contamination routes, thereby emerging as a useful tool available for the management of the hygiene practices in food processing plants.

Indeed, Chaillou et al. (2015) highlighted that with some differences, similar spoilage dynamics can be observed in meat and seafood products. The importance of extrinsic factors related to storage conditions (temperature, type of packaging) was also emphasized: Whereas the microbiota of aerobically stored beef was dominated by *Pseudomonas* and led to more rapid spoilage, LAB were associated with vacuum packaging, and both *Brochothrix thermosphacta* and *Carnobacterium* were identified as SSOs in modified-atmosphere packaging (60% O_2 and 40% CO_2), leading to different spoilage-associated metabolomes (Ercolini et al. 2011). In addition, intrinsic factors, such as the addition of preservatives, may influence the spoilage rate and SSOs involved: Reducing the salt content in raw pork sausages stored under vacuum revealed a decrease of bacterial diversity with the development of a spoilage-associated microbiota (LAB, *B. thermosphacta*, Enterobacteriaceae), leading to more rapid unacceptability of the product (Fougy et al. 2016). Moreover, the addition of sodium lactate and diacetate to sausages led to a dramatic change in the microbiota involved in spoilage: Several LAB and *Pseudomonas* were replaced by *Lactobacillus graminis*, which dominated in samples to which the antimicrobial mixture was added (Benson et al. 2014).

Some studies have aimed to elucidate the possible contamination routes in food handling and processing plants. Beef carcasses contained high microbial diversity and the well-known genera associated with meat spoilage (De Filippis et al. 2013), with differences due to slaughtering practices and occurring at different areas of the carcass (Korsak et al. 2016). De Filippis et al. (2013) suggested that bacteria originally present on the carcass colonize the butchery environment, where they become resident as they are well adapted to meat exudates and low temperature. This resident microbiota represents a primary contamination source for fresh meat (De Filippis et al. 2013). HTS has been widely used to track contamination sources in different types of food processing plants: fresh meat (De Filippis et al. 2013, Stellato et al. 2016); cooked sausages (Hultman et al. 2015); salmon fillets (Møretrø et al. 2016); and ready-to-eat, composite meals (Pothakos et al. 2015). In all cases, a resident microbiota was found and its importance as the primary contamination source was highlighted, as was the need for adequate cleaning and sanitation practices in food-handling environments.

Shotgun-based sequencing approaches are a promising tool for exploiting spoilage dynamics, but to the best of our knowledge, only one study exists. Nieminen et al. (2012) compare spoiled marinated and unmarinated poultry meat and report that the spoilage dynamics were associated with the catabolism of carbohydrates and the marinade was able to inhibit some SSOs, such as *B. thermosphacta*, that produce off-flavors from carbohydrate degradation (Casaburi et al.

Figure 4

Structure of microbial communities in raw and spoiled meat and seafood and related processing environments. Data were extracted from five studies that included meat or seafood products contained within FoodMicrobionet (Parente et al. 2016; data retrieved April 2017; **http://www.foodmicrobionet.org**). (*a*) A bipartite network was produced from rarefied operational taxonomic unit tables. The width of the upper boxes is proportional to the taxon abundance, whereas the width of the ribbons connecting upper (taxa) and lower (food groups) boxes is proportional to the number of sequences assigned to that taxon in each food group. (*b*) Modules of taxa significantly associated with food groups are outlined in red, whereas the taxon relative abundance is indicated by the depth of the purple shading. (*c*) A Venn diagram reveals shared microbial genera among fresh or spoiled meat and seafood and related processing environments. Only genera with abundance greater than 3% are included. Numbers indicate the number of shared taxa.

2014). Although still largely underexploited, metagenomics and metatranscriptomics will aid in the understanding of food spoilage dynamics, highlighting the main microbial pathways leading to sensorial spoilage for the production of off-flavors or other undesirable compounds. In this regard, it is of utmost importance to design studies and molecular and bioinformatics protocols to address spoilage microbes at the strain level, as different strains of the same species can have different effects on the release of spoilage-associated metabolites, and such features are strictly strain specific and dependent on storage conditions (Casaburi et al. 2011, 2014; Ercolini et al. 2010).

The food industry benefits from improved knowledge of spoilage mechanisms. In addition, the effect of applying different technological hurdles may be explored to achieve a more in-depth understanding of the spoilage process and the best ways to limit or retard it.

Omics and Food Safety Issues

Reliable detection, identification, and tracking of foodborne pathogens are fundamental for food safety. Pulsed-field gel electrophoresis and multiple-locus variable number tandem repeat analyses are the currently used subtyping methods for surveillance and detection of foodborne disease outbreaks. However, they have limited discriminatory power for some pathogens, particularly for specific *Salmonella* spp. serovars (Allard et al. 2012, Ranieri et al. 2013, Zheng et al. 2011). In contrast, genome comparison and single nucleotide polymorphism detection have greater discriminatory power, as they are able to cluster the isolates in epidemiologically relevant groups during outbreaks of listeriosis, pathogenic *Escherichia coli* infections, *Campylobacter enteritis*, vibriosis, salmonellosis, and norovirus infections (Baillie et al. 2012, Ronholm et al. 2016). This enables more rapid identification of outbreak-related strains and tracking of the transmission routes, enabling a prompt response by public health authorities. Moreover, the large number of genomes now available from pathogenic microbes is an important resource that can be used in outbreak investigations to track down the geographic origin and food source of a pathogen during or after an outbreak. For this reason, the US Food and Drug Administration promoted a program named GenomeTrakr (**http://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWGS**) for WGS of microorganisms isolated during foodborne illness outbreaks throughout the world to implement a database of foodborne pathogens, and genomes from more than 67,000 isolates (as of April 2017) have already been collected. Genome-sequencing efforts will not only improve outbreak detection and source tracking but also contribute large numbers of foodborne pathogen genomes to public databases, which is useful for data-mining efforts that could provide new insights into foodborne pathogen biology and transmission mechanisms.

In addition, metagenomics approaches may be useful in monitoring the diffusion of foodborne pathogens (Yang et al. 2016) and antibiotic resistance genes (Noyes et al. 2016) along the food chain. Noyes et al. (2016) examined how the antibiotic resistance potential (resistome) is spread in cattle and how these genes are transmitted along the meat processing chain up to retail; they highlighted the importance of this type of study for understanding the mechanisms leading to the dissemination of antibiotic resistance in foodborne bacteria. Already in progress in urban biomes, the mapping of microbial genomes in food-associated environments will help to track the diffusion of antibiotic resistance genes or other important virulence factors, such as toxin production (MetaSUB Int. Consort. 2016). Correlating this information with different sorts of metadata (temperature, humidity, etc.) will help in planning ordinary cleaning practices as well as in the design of novel food processing plants.

Finally, the study of the metatranscriptome of foodborne pathogens can unveil the mechanisms involved in their response to common antimicrobial agents (Casey et al. 2014, Visvalingam et al.

2013) or to the conditions normally used during food processing and storage (Fink et al. 2012, Goudeau et al. 2013), which will help in understanding how to prevent their dissemination and development.

FOOD MICROBIOME, BIOINFORMATICS, AND DATA ANALYSIS

The typical output of a metagenomic study consists of millions of reads that can be considered at the same time, which is both the greatest potential and the main drawback of HTS. Bioinformatics analysis is required to translate sequences into meaningful data. The greater the number of reads, the greater the computational power required for data analysis, which often cannot be supported by a standard desktop computer. In addition, appropriate bioinformatics skills are necessary. Standardized pipelines for the analysis of data arising from amplicon-based sequencing have been developed, making this type of analysis more accessible, even to biologists lacking a bioinformatics background. Although the single steps of a typical amplicon-based analysis are the same, high variability exists in the possible options and parameters, which can impact the final result. Therefore, there is a need for standardized data analysis platforms addressing all steps of the workflow, from sequence quality analysis to OTU selection and taxonomic assignment. Recently, R packages for standardized analysis have emerged (Callahan et al. 2016, McMurdie et al. 2015). In addition, although the use of descriptive tools for α- and β-diversity analyses is widespread, inferential tools are less common in food microbiology, but microbial association network analysis may offer significant advantages in detecting ecological and biological associations in food microbial communities (Layeghifard et al. 2016).

More variability in the possible approaches to shotgun data analysis exists, which still requires computational capabilities and knowledge in bioinformatics that do not commonly occur in all microbiology laboratories. The typical data analysis workflow includes short-read assembly, gene prediction, and annotation through mapping to a specific database. Nevertheless, assembly is not always possible (or satisfying) for low-coverage genes or genomes. In addition, this step becomes more computationally intensive with an increasing number of sequences, which is sometimes infeasible. Bioinformatics tools for HTS data analysis are constantly evolving and improving. A new application offers the possibility of extracting strain-level information directly from the metagenome (**Figure 5**). Common pipelines based on genome assembly often fail to reconstruct genomes for less-abundant species and strains. Therefore, new tools have been developed to enable pangenome-scale analysis directly from short reads and thereby overcome this limitation. StrainPhlAn (Truong et al. 2017) extracts species-specific, high-polymorphic marker genes from metagenomics reads, aligning them to a database of reference genomes and allowing a strain-level comparison of different samples. Moreover, PanPhlAn (Scholz et al. 2016) enables users to functionally characterize the strains present in a metagenomics sample, aligning reads to a species pangenome database built using available reference genomes.

The possibility of a strain-resolution analysis based on HTS of rRNA, species-specific gene amplicons was also exploited but with unsatisfactory results (De Filippis et al. 2014, Ricciardi et al. 2016). Strain-level dissection of metagenomes promises to overcome these limitations.

Nevertheless, when metagenomics data are not available or cannot be generated/analyzed, further bioinformatics tools may be employed to analyze amplicon sequences to extract information at the subgenus level. For example, oligotyping promises species- or even strain-level discrimination within a group of highly similar reads arising from 16S rRNA gene amplicon sequencing. It decomposes a given taxon into high-resolution units (oligotypes) by considering the nucleotide positions identified as the most information rich (high-entropy positions) (Eren et al. 2013). Although not comparable to the information obtainable through strain monitoring based

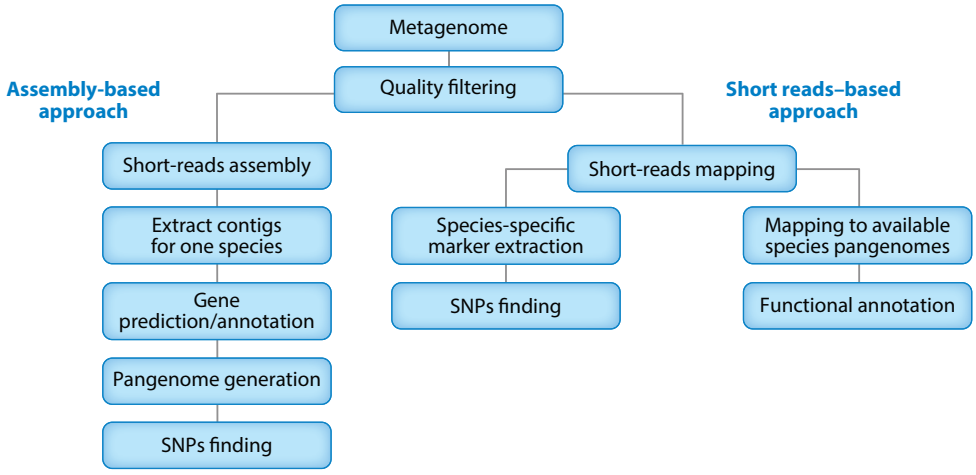

Figure 5

Different possibilities available for pangenomics analysis directly from metagenomics data. Abbreviation: SNPs, single nucleotide polymorphisms.

on a shotgun approach, oligotyping may be useful to obtain interesting information at a subgenus level, which is not always possible with the typical analysis based on de novo OTU clustering of amplicon reads. Oligotyping was successfully used to find ecologically meaningful differences within the most abundant genera of the human gut microbiota (De Filippis et al. 2016b, Eren et al. 2015) and in environmental samples (Turlapati et al. 2015), but to the best of our knowledge, only one application in the food-related environment exists. Stellato et al. (2017) determined that the same oligotypes of *Pseudomonas* spp. were present in dairy and meat processing environments and in related food samples, although their relative abundance differed according to the food matrix considered. This highlights the presence of species- and possibly strain-related responses to the selective pressures existing in different foodstuffs.

ISSUES AND LIMITATIONS

Like all molecular methods used for microbial community description, HTS suffers from biases associated with the procedure used for NA extraction. This issue becomes particularly important in HTS studies, in which a quantitative description of the microbiota is expected. In fact, differences in cell wall organization may cause preferential NA extraction from some taxa at the expense of others, altering the results obtained for the microbial community under study (Keisam et al. 2016, Quigley et al. 2012a). Also, the PCR step required for amplicon library preparation may lead to preferential amplification of specific taxa: The selection of the primer set may strongly influence the qualitative and quantitative descriptions of the microbiota obtained (Bokulich & Mills 2013b, Cruaud et al. 2017, Sergeant et al. 2012). This issue is particularly relevant for studies of fungal populations. The most frequently used target for fungi is the internal transcribed spacer (ITS). Nevertheless, the uneven ITS length among fungal species may lead to preferential amplification of shorter fragments, further distorting the results (De Filippis et al. 2017a). Therefore, efforts should be made to promote the use of different targets in HTS-based studies of fungal populations (De Filippis et al. 2017a), such as 26S and 18S rRNA genes (De Filippis et al. 2017b, Garofalo et al. 2015, Minervini et al. 2015, Stellato et al. 2015, Wang et al. 2015). For all these reasons,

taxonomic binning of metagenomics reads may produce more reliable results (Liu et al. 2011). An additional problem associated with the amplification of ribosomal genes is the operon copy number, which differs across taxa, distorting the quantitative estimation (Kembel et al. 2012). Therefore, single-copy target genes, such as *recA*, *radA*, *rpoA*, *rpoB*, and *gyrB*, have been suggested as alternatives (Kembel et al. 2012, Renouf et al. 2006, Vătrovský et al. 2013), but their use is still limited due to the absence of databases with sufficient coverage.

Although offering remarkable advantages, the widespread application of shotgun-based approaches is still in the developmental phase for several reasons. First, shotgun-based studies require more reads per sample, increasing costs. Therefore, these types of studies often focus on only a few samples, thus limiting the power of the analysis. Furthermore, because DNA (or complementary DNA after a reverse transcription step) fragmentation is required for library preparation, the integrity of the extracted NAs is a crucial factor. Specific types of samples, such as meat and seafood products, present the additional issue of host NA contamination, which may subtract a substantial number of sequences from the analysis. Finally, when mRNA is the desired target, further precautions must be taken to deplete the rRNA concentration (usually more than 90% of total RNA) and block RNase activity, thereby freezing gene expression at the moment of sampling.

A metagenome predictive tool, named PICRUSt, has been developed (Langille et al. 2013) that promises to predict the functional potential of the bacterial community present in a sample based on 16S rRNA gene amplicon data. Basically, it relies on the information present in a genomic database and thus cannot guarantee reliable results for environments where only a few genomes are available. This pipeline is currently usable only for bacteria (and not for fungi) and obviously cannot take into account strain-level differences; however, with these limitations in mind, PICRUSt may still provide useful information at no cost when a real metagenome is not available.

The bioinformatics pipeline followed for data analysis must be considered a crucial step in both amplicon- and shotgun-based studies, as the different software and parameters employed and the reference database used may strongly influence the final results (May et al. 2014). The choice of a well-curated and up-to-date database is a critical factor, particularly for fungi, as available fungal databases are considered less curated than bacterial databases (Tedersoo et al. 2011). The ITS is considered the most complete fungal database, but as stated above, this target may not be the best choice because of length heterogeneity (De Filippis et al. 2017a). Therefore, food microbiology would greatly benefit from the improvement of available genomics databases, which should be enriched with genomes from foodborne microbes, and studies aimed at developing food-specific gene catalogs, as recently proposed for cheese, would be invaluable (Almeida et al. 2014). As discussed above (see section Food Microbiome, Bioinformatics, and Data Analysis), bioinformatics analysis can still be considered the bottleneck of HTS-based studies. Indeed, data analysis requires bioinformatics abilities not always available in a microbiology laboratory.

FUTURE STEPS

After one decade of the widespread use of HTS of the microbial ecology of food, public databases have accumulated a large amount of data, mostly arising from amplicon-based studies. However, most of the amplicon-based results are basically descriptive, and although they have a greater resolution, limited new information has been obtained. Despite this, we have an unprecedented opportunity for data sharing. Sequence data made available in public databases [e.g., the Sequence Read Archive of the National Center for Biotechnology Information (**http://www.ncbi.nlm.nih.gov/Traces/sra**) and the European Nucleotide Archive of the European Bioinformatics Institute (**http://www.ebi.ac.uk/ena**)] may be used in comparative meta-studies; a good example is FoodMicrobionet (Parente et al. 2016), mentioned above.

Although still underexploited in food microbiology, shotgun metagenomics and metatranscriptomics promise to provide new insights into the functions of food microbial consortia, helping us to understand the mechanisms of community assembly and their drivers, including the influence of important environmental factors. This knowledge may be easily transferable to microbial consortia that inhabit more complex environments, making foods tractable models for dissecting microbial assemblage mechanisms (Wolfe & Dutton 2015). Moreover, with the development of new bioinformatics tools for data analysis (see section Food Microbiome, Bioinformatics, and Data Analysis), recovering draft genomes directly from metagenomics reads is now possible (Marx 2016). This is particularly relevant in food microbiology issues, as many technologically relevant traits (Douillard & De Vos 2014, Hao et al. 2011, Sun et al. 2015) and spoilage-associated activities (Casaburi et al. 2011, 2014; Ercolini et al. 2010) are strain dependent. This is a groundbreaking opportunity not only for epidemiologic studies but also for quantitative strain monitoring of microbial species of interest during fermentative or spoilage processes, directly in the food matrices. It will be helpful to understand the functional potential of foodborne microbes, how they respond to technological parameters, and how to exploit or defeat them.

Omics technologies have revolutionized food microbiology laboratories and our approach to the study of a microbial community. The use of a combination of molecular approaches will enable in the near future the unveiling of complex interactions that occur within microbial consortia in food environments, increasing our understanding of how to exploit invaluable microbial resources to ensure process efficiency as well as food quality and safety.

SUMMARY POINTS

1. HTS of taxonomically relevant genes is the most exploited application in food microbial ecology. Although these studies provide higher resolution and potential insight into microbe–process associations, they are basically descriptive and do not add appreciably to our knowledge of food microbial communities.
2. Shotgun metagenomics and metatranscriptomics are still underexploited. Nevertheless, the few studies available have revealed that they elucidate the complex activities existing in food environments and increase understanding of how technological parameters can be modulated to affect microbial functions.
3. Reduced sequencing costs increased the WGS of several microbial strains from the same species, making them available for comparative studies and highlighting the presence of strain-specific traits. Genomic databases are a valuable resource for epidemiologic studies.
4. Extracting genomes of dominant strains directly from metagenomics reads enables quantitative strain monitoring directly in food matrices, providing insight into how different strains respond to abiotic factors.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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