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MINIREVIEW

High-Throughput Sequencing and Metagenomics: Moving Forward in the Culture-Independent Analysis of Food Microbial Ecology

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Following recent trends in environmental microbiology, food microbiology has benefited from the advances in molecular biology and adopted novel strategies to detect, identify, and monitor microbes in food. An in-depth study of the microbial diversity in food can now be achieved by using high-throughput sequencing (HTS) approaches after direct nucleic acid extraction from the sample to be studied. In this review, the workflow of applying culture-independent HTS to food matrices is described. The current scenario and future perspectives of HTS uses to study food microbiota are presented, and the decision-making process leading to the best choice of working conditions to fulfill the specific needs of food research is described.

The study of the microbial ecology of foods has dramatically changed. Functional genomics, transcriptomics, proteomics, and metabolomics have all been applied to understand the behavior of microorganisms in foods (1). In addition, for the foreseeable future, food microbiologists are unlikely to be able to do without "detectomics": a major priority is to develop and optimize molecular methods for the detection, reliable identification, and monitoring of food-associated microorganisms (2). Culture-independent analyses arose to overcome the limitations of the classical culture-based approach and have been extensively used in food microbiology (3–6). The scope of microbial analysis can depend on the specific food, and the target microbes can be (i) pathogens, (ii) spoilage associated, or (iii) (potential) starters and beneficial microorganisms. Such microbial populations deserve attention because of their role in food contamination, spoilage, or fermentation (i.e., food production). The study of microbial diversity can now be achieved by using high-throughput sequencing (HTS) approaches after direct nucleic acid extraction from the matrix to be studied. Several next-generation sequencing (NGS) technologies have been developed (7, 8). Descriptions of the various NGS systems and platforms, as well as their advantages and disadvantages, have been extensively reviewed (9–13). Workflow, limits, and perspectives in applying culture-independent HTS to study food microbiota are presented in this minireview.

WORKFLOW FROM FOOD TO SEQUENCES

An overview of the possible applications of culture-independent HTS in food microbial ecology is shown in Fig. 1. The occurrence and abundance of microbes and genes in a given food ecosystem can be evaluated by studying the microbiome, which refers to the microorganisms and their genomes in the environment in question. The study of microbiota is instead based on rRNA amplicon sequencing and can give the proportions of taxa within a food sample. In addition, HTS of specific target genes can provide strain monitoring in food samples (Fig. 1). The use of rRNA amplicon sequencing to study microbiota is the most common HTS application in microbial ecology and the only application exploited in foods. This entails the analysis of amplicons arising from a complex mix of microbial genomes directly extracted from a food sample. The targets for such analyses are of course genes of taxonomic interest, with the 16S rRNA gene being the most widely used for bacteria (9, 14). rRNA amplicons from DNA/RNA directly extracted from foods are sequenced, and the sequences are compared to reference databases to identify the operational taxonomic units (OTUs). The number of sequence reads identified with the same OTU is calculated, and a quantitative estimation is then given on the occurrence of each OTU in the sample analyzed.

HTS APPLICATIONS IN FOOD

rRNA amplicon HTS has been applied mainly to study food fermentation or food spoilage (Table 1). In both cases, the keywords are the structure of the microbiota and its evolution in space and time.

The structure of the microbiota by rRNA-based HTS defines the microbes populating a specific food. This helps characterize the final products to determine the proportions between fermenting microorganisms and versus microbial contaminants. A comprehensive survey of about 60 Irish soft, semihard, and hard cheeses was recently performed by HTS to screen for differences in bacterial diversity according to cheese type, milk, and production technology. The structures of cheese microbiota varied according to the animal origin of the milk and the ingredients used and also differed between pasteurized and raw milk (15). It was shown that the *Lactobacillus* populations increased in hard cheeses compared to those in soft cheeses, indicating an effect of the level of cheese maturation on the development of lactic acid bacteria (LAB). In addition, high salt content in some cheeses resulted in the absence of *Leuconostoc* and *Pseudomonas*. Moreover, the inclusion of ingredients such as herbs, spices, or seaweed impacted microbial composition (15). Microbial diversity can also give information on the mode of food production. Indeed, the more traditional the manufacturing process, the more complex the microbial community in the food. In contrast, industrially obtained foods are characterized by more-simple microbial consortia (16–18).

The spatial distribution of microbes in foods is also a very interesting issue. Using other culture-independent tools, it was

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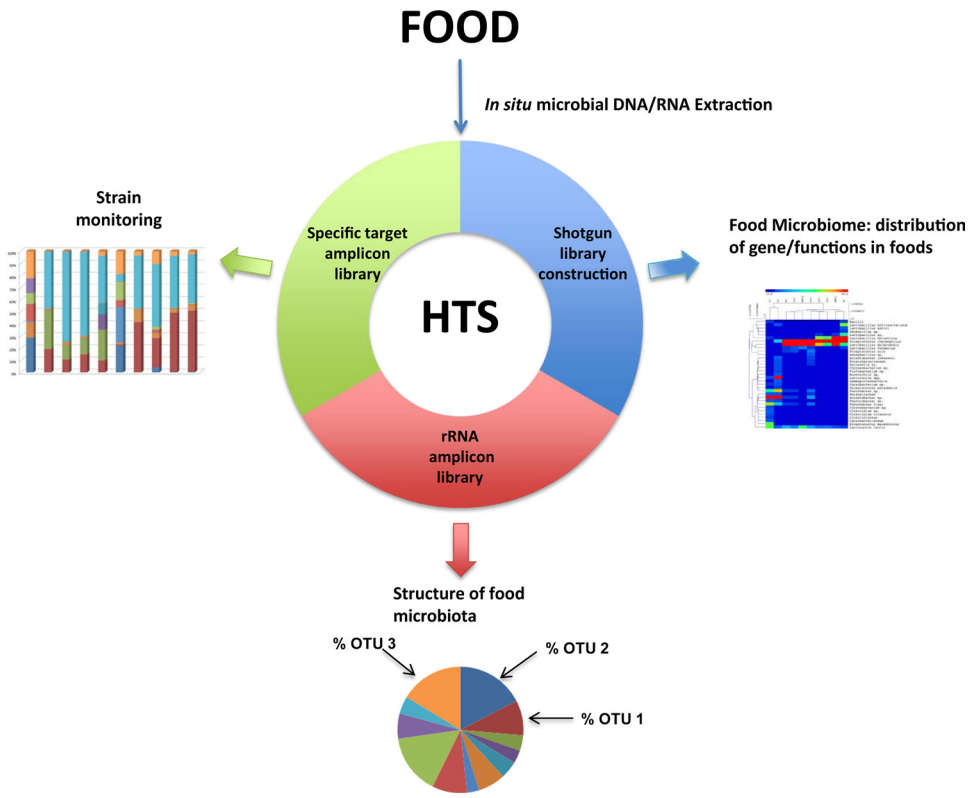

FIG 1 Culture-independent HTS applications to study food microbiota and possible outputs in a general workflow.

shown that foods with a complex structure can host a heterogeneous distribution of microorganisms within their different parts, such as the crust, veins, and core in a blue cheese (19). Location of microbes in food has important consequences because it affects ripening, flavoring, protection, spoilage, and ecological dynamics in each part of the food. HTS is suited to such investigation, providing in-depth assessment of the location of different microbes across food matrices. Indeed, HTS has been used to highlight a significantly different structure of the microbiota in cheese and cheese rinds (15).

TABLE 1 Working conditions used and taxonomic resolution achieved in the culture-independent HTS analysis of food microbiota targeting the 16S rRNA gene

Food sample source[a]	Variable region	Amplicon length (bp)	Sequencing platform	Taxonomic resolution	Database used	Reference(s)
Botrytized wine fermentation	V4	~150	Illumina GAIIx	Family/genus	RDP	30
American coolship ale fermentation	V4	~150	Illumina GAIIx	Family/genus	RDP	22
Fermentation of pearl millets	V3	~180	454 FLX	Genus	RDP	55
Fermented seafood	V3	~180	454 FLX	Genus	Greengenes	56
Irish cheeses	V4	~250	454 FLX	Genus	NCBI	15
Kefir and kefir grains	V4	~250	454 FLX	Genus	NCBI	57
Fermented soybean (doenjang)	V1-V2	~300	454 FLX	Genus/species	RDP	16
Fermented soybean (cheonggukjang)	V1-V2	~300	454 FLX	Genus	RDP	17
Fermented red pepper condiment (kochujiang)	V1-V2	~300	454 FLX	Genus/species	RDP	18
Fermented fish (narezushi)	V1-V2	~300	454 FLX	Genus/species	RDP	58
Fermented fish (narezushi) and intermediates of production	V1-V2	~300	454 FLX	Genus	RDP	59
Kefir	V1-V2	~300	454 GS 20	Genus	RDP	60
Oscypek cheese and intermediates of production	V5-V6	~300	454 FLX	Genus	Silva	61
Fermented rice bran mash (nukadoko)	V6–V8	~400	454 FLX	Genus/species	RDP	62
Danish raw milk cheeses and intermediates of production	V3-V4	~450	454 FLX	Genus	RDP	21, 24
Water buffalo mozzarella cheese and intermediates of production	V1–V3	~500	454 Junior	Species	Greengenes	20
Beef in different storage conditions	V1–V3	~500	454 FLX	Genus/species	NCBI	23, 63
Korean rice beer fermentation	V1–V3	~500	454 FLX	Genus	RDP	64
Fermented soybean (meju)	V1–V3	~500	454 FLX	Genus/species	RDP	65

[a] Examples are ordered according to increasing amplicon length.

Understanding how microbiota evolve over time is a priority in studying the microbial ecology of foods. Changes in microbial populations provide useful information to follow natural fermentation dynamics, monitor the fate of starter or adjunct cultures, or observe the shifts in spoilage-associated populations according to food storage conditions. HTS was recently used to examine microbial succession during traditional mozzarella cheese manufacture, and the microbiota associated with this dairy production was shown to be not as complex as previously thought (20). In Danish raw milk cheeses, bacteria from the starter culture dominate raw milk cheese (21). In the microbiota involved in brewing American coolship ale, *Enterobacteriaceae* were found to dominate at the initial fermentation stages while *Lactobacillales* and yeasts took over in the subsequent phases (22). In this example, HTS analysis clearly showed the stability of autochthonous bacteria in long processes and highlighted the role of resident microbiota (that of the brew house in the specific case) in fermentation.

Monitoring changes during fresh food storage is also of great importance. Using the sensitivity of HTS, it was shown that the initial microbiota of beef changes dramatically upon storage of the same beef for 40 days in completely different packaging conditions (23). Indeed, *Brocothrix thermosphacta* and *Pseudomonas* sp. dominated in the first and second stages of air storage, respectively, while *B. thermosphacta* and *Carnobacterium divergens* developed in the first and second periods of modified-atmosphere packaging (MAP) storage, respectively. More OTUs belonging to the lactic acid bacteria (LAB) group were observed during vacuum pack storage, while when meat was stored in nisin-activated antimicrobial packaging, 95% of the OTUs were identified as *C. divergens* in the final stages of storage (23). Detection of such changes can be invaluable to plan appropriate storage conditions for food products so as to inhibit specific microbial populations.

Although testing foods for the presence of food-borne pathogens by using culture-independent approaches is problematic due to several methodological limits, HTS can be useful to reveal contamination by pathogenic microorganisms or to monitor their reduction upon specific food treatments. Although the specific strain was not necessarily pathogenic, an *Escherichia coli* strain deliberately added to milk was found to be metabolically active (by cDNA pyrosequencing) for up to 7 days of ripening of Danish cheeses while it decreased in subsequent stages affected by fermentation (24).

Food-borne pathogens are very often targeted to define their fate in food processing, fermentation, or storage. To this aim, many different group-, genus-, and species-specific PCR assays have been developed and used for the detection of pathogens in food (25, 26). In all cases, once a set of specific primers has been designed, the PCR assay must be checked for cross-reaction and false positives with nontarget microorganisms. The HTS approach has been successfully used to validate a *Campylobacter*-specific PCR assay and to demonstrate the specificity of the PCR assay against a complex set of untargeted bacteria in a natural environment (27).

Finally, since little is known about the ecological role of bacteriophages in fermented foods, shotgun sequencing of viral DNA can also be used to define the viral communities in foods, as recently shown in fermented shrimp, kimchi, and sauerkraut (28).

PROS AND CONS: CAN WE TAKE HTS BEYOND RESEARCH PURPOSES?

HTS was conceived and is currently employed for research laboratories. To evaluate the possibility of scaling up the analysis for the benefit of food industry requirements, strengths and weaknesses need to be analyzed (Fig. 2). None of the culture-independent methods currently employed to study food products has a throughput comparable to HTS. Thousands of sequences available from HTS analysis can be readily analyzed to ensure swift, reliable identification of the majority of microorganisms occurring in food samples. Depending on the desired level of sample coverage, many food samples can be sequenced at the same time, saving much time compared to the approaches currently used. In addition, when microbiomes are studied by shotgun library sequencing, insights into microbial activities can be obtained from the sequences of microbial genes present in the original food sample, which offers important advances in studying microbial ecology of foods. The HTS approach entails a safer bench activity with reduced exposure to unsafe reagents used, for example, for electrophoresis. Moreover, with some sequencing technologies or by using automated liquid handlers, there is an almost negligible contribution of the operators and much bench time is saved in the laboratories. However, the drawbacks of HTS include the need for bioinformatic analysis of data and, depending on the choice of the specific working conditions, the cost of analysis per sample (Fig. 2). The final output of HTS is thousands of sequences that need to be studied in order to translate them into useful information for food-associated microbial ecology. The bioinformatics part of the study cannot be performed by any laboratory worker: managing large numbers of sequences does not just require simple "blast" procedures that many students have learned in molecular biology laboratories. Skilled bioinformaticians must be specially trained for this activity, and therefore, HTS technologies cannot just be acquired in a laboratory and used immediately. Costs of analysis are decreasing significantly as a result of new lower-cost technologies becoming operative and competition between the different HTS platform suppliers. However, the initial cost of the equipment is rather high, especially compared with the cost of electrophoretic equipment used for traditional culture-independent approaches. In light of the above-mentioned considerations, it is unlikely that the food industry will readily acquire equipment and know-how to use HTS analyses of foods. The food industry will probably not need routine use of the technology and will therefore call on external services to process their own food samples under specific requirements and for specific project needs.

PLANNING SAMPLE COVERAGE AND TAXONOMIC RESOLUTION TO SET WORKING CONDITIONS

With the HTS approach based on rRNA gene sequencing, the structure of a microbial community from food can be described by determining a large number of sequences for each sample analyzed. As stated above, this can be achieved through different technologies that do not give exactly the same result in terms of sample coverage and taxonomic resolution. Coverage is given by the number of sequences retrieved for a particular sample. In theory, the higher the better; analyzing tens of thousands of sequences for a single sample ensures thorough determination of the structure of the microbial community, with the possibility of highlighting the presence of very minor OTUs. However, determining a very large number of sequences per sample can be ex-

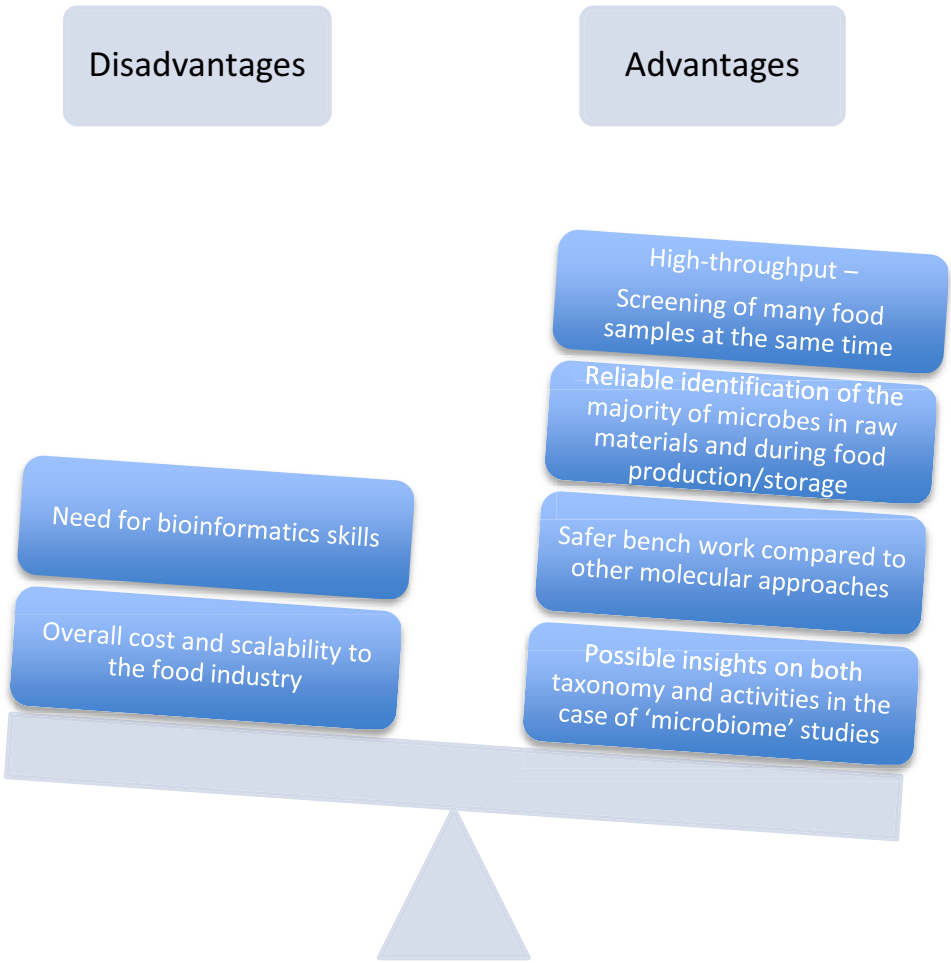

FIG 2 Advantages and disadvantages of the use of HTS to study food-associated microbial ecology.

pensive and is sometimes redundant. Rarefaction analysis of sequencing data (29) can be of help to decide a numerical threshold of sequences that may be informative for the type of sample under study. This is a basic decision that is very critical in planning HTS of food samples. Examples of rarefaction plots from 16S-based HTS analyses of foods are depicted in Fig. 3. Typically, a rarefaction curve shows the variation in the number of OTUs identified at a given percentage of identity as a function of the number of sequence reads obtained per sample. Ideally, an optimal coverage to describe the microbiota is identified by the plateau of the curve, which indicates that increasing the number of reads does not change the number of OTUs that can be determined. These trends depend upon the level of the diversity of each particular sample. In the case of foods, samples with a complex microbiota, such as raw milks or any other "raw" material, are likely to have a large number of OTUs and will need a good number of reads to be properly characterized. On the other hand, for pasteurized foods or fermented foods or when starter cultures are added, and where the microbiota is less complex because a selection of OTUs has taken place and a limited number of species occur, even a small number of sequences can be enough to properly define the structure of the community. As reported in Fig. 3, a large number of sequence reads are needed to study raw milks for mozzarella cheese production, whereas about 2,000 sequence reads will suffice to assess the microbiota of the fermented curd or mozzarella cheese samples, as the level of microbial diversity will be lower after fermentation (Fig. 3A). Similarly, fresh meat will require more sequences than spoiled meat samples (Fig. 3B): the diversity of meat microbiota will be reduced during aerobic spoilage in which few selected microbial species will grow, outcompeting the initial complexity of the microbiota of the fresh meat ecosystem (Fig. 3B).

The data on food products reported in the literature include a variable number of reads per sample, ranging between 100 and >10,000 depending on the specific study and sample analyzed. However, not all of the studies report a rarefaction analysis and it is not always possible to understand how deep the sample coverage has been. For example, coverage obtained from about 1,000 reads was satisfactory to determine the microbiota of some fermented soybean products (16, 17). Most samples of soft, semi-hard, and hard Irish cheeses were adequately covered with slightly more than 1,000 reads, while cheese rinds, which have a more complex microbiota, required more sequences to be adequately studied (15). Finally, adequate coverage was reached beyond 5,000 sequences per sample of fermenting must for fermented alcoholic beverages (22, 30).

Taxonomic identification is another issue to be addressed when approaching the study of food microbiota by HTS. Foods are microbiologically complex matrices but are not as rich in taxa

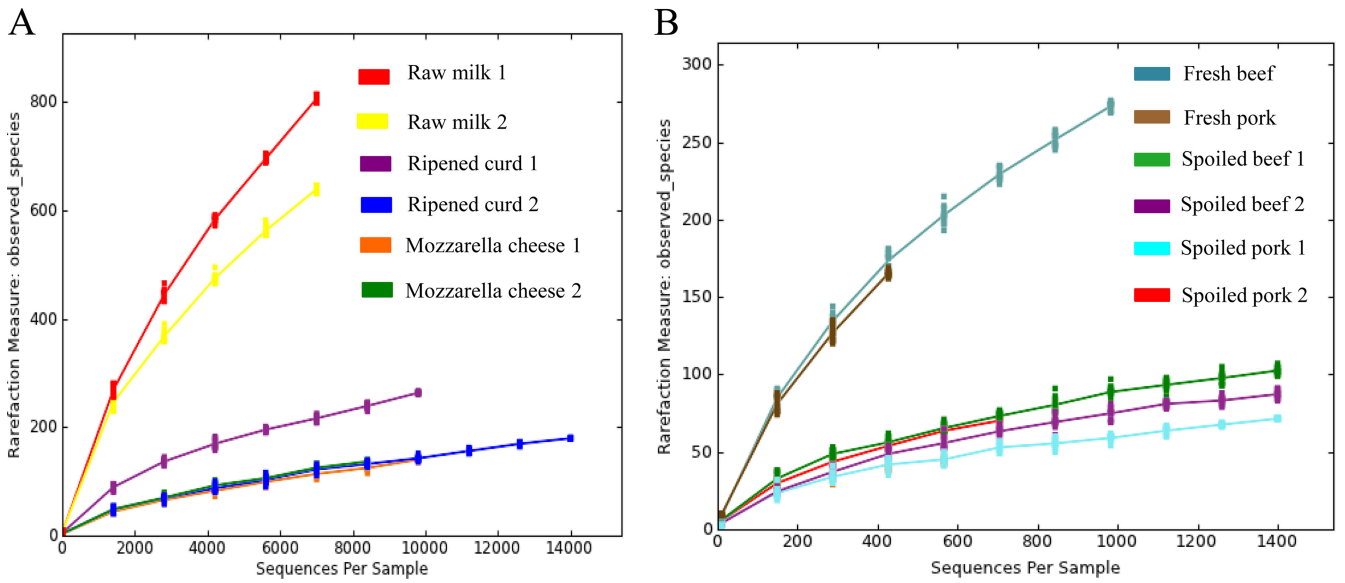

FIG 3 Examples of rarefaction curves reporting the number of observed OTUs as a function of the number of sequence reads. Data were retrieved from HTS analyses of samples of mozzarella cheese and production intermediates (20) (A) and fresh and aerobically spoiled pork and beef (D. Ercolini, F. De Filippis, and A. La Storia, unpublished data) (B).

as other environmental samples, such as soil, wastes, feces, etc., in which microbial diversity can be fruitfully investigated at the genus level or even larger hierarchical taxonomic ascriptions, such as family, order, class, or phylum. The taxonomic resolution required in foods can vary depending on the purpose of the study. For example, genus-level monitoring of microbial diversity in a food during fermentation or storage can be used when considerable changes in the structure of the community are expected. However, in many cases, species-level identification is needed to obtain useful information in food. In a typical example, in population changes during cheese ripening, there is often a succession of *Lactobacillus* species in which thermophilic species are responsible for the initial fermentation while mesophilic lactobacilli take over during cheese ripening. Similarly, in other fermented products, such as sourdough or fermented meats, many different species of the same genus can occur and take turns during fermentation. In such cases, an HTS study at the genus level is not informative and species assignment should be the target of the analysis. For this purpose, long sequence reads including more-variable regions of the 16S rRNA gene are required for accurate assignment. However, even with long reads, the 16S rRNA gene is not always heterogeneous enough for species discrimination in food-related bacteria, as observed in *Pseudomonas* spp. (31, 32). Table 1 summarizes the variable 16S regions, amplicon length, the HTS platform used, and the taxonomic resolution achieved in research using HTS to study food microbiota. Fragments of 16S rRNA from 150 to 500 bp, including one to three different variable regions, were employed. It can be clearly noted that the longer the size of the amplicons sequenced, the more detailed the taxonomic assignments that can be obtained (Table 1). The choice of amplicon length and sample coverage is a critical variable and should depend on the specific food and on the scope of the project. A good number of reads is advisable for sample coverage, and a satisfactory fragment length is desirable for in-depth, reliable taxonomic identification.

CRITICAL ISSUES

Since culture-independent HTS analysis of microbiota is considered quantitative, all the possible issues that can lead to an alteration of the original proportion of microbial cells (or DNA extracted therefrom) in a specific food sample must be avoided because it may lead to unreliable pictures of the microbiota. In theory, the approach is quantitative because there will be proportion between abundance of a specific microorganism in the food, quantity of nucleic acid extracted, quantity of amplicons obtained, and the number of sequences gained belonging to that specific microorganism. Therefore, the number of sequences obtained is ultimately proportional to the abundance of the microorganism in question. All possible efforts thus need to be made to keep the above-mentioned proportion unaltered.

Sampling and sample handling are frequent problem sources regardless of the analytical approach used (33). Once the sample is collected, altering the proportion of the microorganisms both before and after nucleic acid extraction must be avoided. Any such alterations would result in appreciable changes in the ratios between sequence numbers and OTU abundance, with doubtless oversights in the estimation of the proportions of microbial populations in the original food sample. As far as sample handling is concerned, aerobic or anaerobic storage, transport, freezing, or chilling may affect the development of the microorganisms in the food by altering the number and species to be detected.

A further source of variability may be the nucleic acid extraction. Not all microbial species have the same sensitivity to lytic agents, with the differences being due mainly to the organization of the cell wall. This affects the analyses based on *in situ* nucleic acid extraction, since a high yield in pure DNA/RNA is desired, as well as the detection of all the species occurring in that environment. The more complex the matrix, the more difficult it is to obtain good extraction and to get rid of all the impurities that can negatively affect the PCR amplification step. The case of food ma-

trices is particularly awkward; the presence of natural constituents, such as lipids, proteins, carbohydrates, and salts, may render extraction very hard, and some of these molecules can persist until the end of the extraction and be found in the extract, where they might act as PCR inhibitors (34). It is thus very important to choose an extraction procedure that is most efficient and provides templates from all the microbial entities occurring in the original sample. Some examples of optimization of DNA extraction from food matrices can be found in the literature (35, 36).

The PCR itself may be a source of bias in culture-independent analysis of food samples. Differential or preferential amplification of rRNA genes by PCR is an acknowledged problem (37, 38). Preferential amplification would determine that the abundance of the OTUs detected may not exclusively reflect the proportion of the microorganisms in the original sample.

The HTS approach is greatly influenced, indeed driven, by analysis of sequences (39). Several open-source programs are available for processing 16S amplicon HTS data (40–42). The use of such bioinformatic tools in HTS-based microbial ecology has been reviewed elsewhere (43, 44). The accuracy and reliability of the final determined structure of the food microbiota depend very much on the quality of the reference database used to assign the taxonomy (45–47). Various databases are available for prokaryotes (47–50), with all containing reliable-quality 16S rRNA gene sequences. There is currently less in-depth coverage of fungi: although they can be identified on the basis of internal transcribed spacers (ITS) and small and large ribosomal subunits, the public databases often have poor-quality sequences and curated databases have limited coverage (45, 46). In addition, in the specific case of ITS, amplicon length unevenness can promote preferential amplification of shorter sequences, making it necessary to optimize the target regions to be analyzed (51). This is particularly inconvenient for food analysis by HTS, which would benefit from application to fungi given the extreme importance of yeasts and molds in fermentation, ripening, and spoilage of food products.

SPACE FOR FURTHER EXPLOITATION AND FUTURE PERSPECTIVES

A promising application of HTS in food microbiology is the possibility of strain typing and monitoring. Strain typing is of great importance for in-depth investigation of microbial dynamics in foods. Indeed, strain monitoring can address many important questions. For example, some microbial species play a major role in triggering food spoilage and subsequent dynamics (52). However, is there a dominant strain population that drives spoilage? The same question applies to species of starter cultures used for food fermentation. Does a starter "strain" actually dominate the ecosystem during fermentation? In addition, foods contaminated by pathogens may benefit from strain-specific investigation of the microbiota that may reveal the occurrence of more than one pathotype in the case of correlated episodes of infection/intoxication and may contribute to performance of concomitant molecular risk analysis. For these purposes, culture-independent strain typing by HTS could be performed by studying key genes that have significant intraspecies heterogeneity (Fig. 1).

Another interesting application of HTS may be the study of microbiomes from foods, determining the proportion of all the microbial genes in a given sample. To the author's knowledge, only two studies reported metagenome shotgun sequencing from food. One investigated the microbiota of marinated and unmarinated broiler meat (53), and the other reported the metagenome sequencing of kimchi, a traditional Korean fermented food (54). Although the studies of metagenomes from food have great potential, a current limitation is the database availability due to the many genomes of food-related bacteria still to be sequenced. In addition, metagenomic data often include a large proportion of genes encoding basic cell functions that are not always related to specific activities of interest for a particular food microbiota, such as key enzymes for flavor compound production, toxin synthesis, or specific amino acid degradation. Detection by sequencing of such activities will probably be hampered by the detection of sequences of genes encoding basic functions. It is thus desirable that, in the near future, enrichment protocols be studied and developed for *ad hoc* sequencing of discrete parts of the metagenomes and metatranscriptomes in order to allow monitoring of changes in abundance, not only in species diversity but also in specific microbial activities.

CONCLUDING REMARKS

Although all the limitations must be carefully considered, HTS has the potential to become a powerful tool for the culture-independent study of food microbiota. Improvements are still needed in order to extend the range of the microbiota to be identified and improve the potential extent of taxonomic identification, which is important for food microbiota. HTS is destined to be complementary to other metasciences to ascertain the specific roles of bacteria in food, hopefully leading in the near future to a clear examination and understanding of the microbe-driven changes in foods.

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