Food Microbial Ecology in the 'Omics' Era

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Introduction

The total number of microbial cells on earth is estimated to be about 10^{30} (Turnbaugh and Gordon, 2008), but 99% of all microorganisms in almost every environment on earth remain, as yet, uncultured (Amann et al., 1990; Curtis et al., 2002). Culture-independent analyses arose to overcome the well-known limitations of the classical culture-based approach (Ercolini, 2013): since most of the microorganisms are not able to grow in the common laboratory media, when we use the classical cultivation methods, we select only a part of the microbiota (those microbes which are able to grow in the medium used), and we can get only a partial picture of the microbial diversity of that environment. For this reason, in the past 20 years, we moved to the culture-independent approach, which allows analyzing the microbiota by using the nucleic acids directly extracted from the matrix, without applying any selection. Culture-independent techniques completely revolutionized our way of studying microbial ecology and we started to consider microbial populations as consortia of microbes, leading to a real 'cultural' evolution (Cocolin and Ercolini, 2015). The advent of next-generation sequencing technologies in 2004 provided unprecedented sampling depth compared to traditional culture-independent approaches, such as denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism analysis, or Sanger sequencing of 16S rRNA gene clone libraries. The possible applications of high-throughput sequencing (HTS) in food microbial ecology are summarized in Figure 1. HTS studies in microbial ecology can be grouped into two fields: target-gene surveys (so-called 'amplicon metagenomics'), based on the sequencing of libraries of amplicons of a gene of interest, and shotgun metagenomics, where libraries of randomly isolated DNA fragments are sequenced (Figure 1). In the first case, a polymerase chain reaction (PCR) step is performed after total DNA extraction (RNA has to be retrotranscribed to complementary DNA), in order to select the gene to be sequenced. In the shotgun approach, no PCR selection is performed and total DNA or cDNA are fragmented and directly sequenced. Moreover, HTS is uniquely quantitative: the number of reads obtained for each operational taxonomic unit

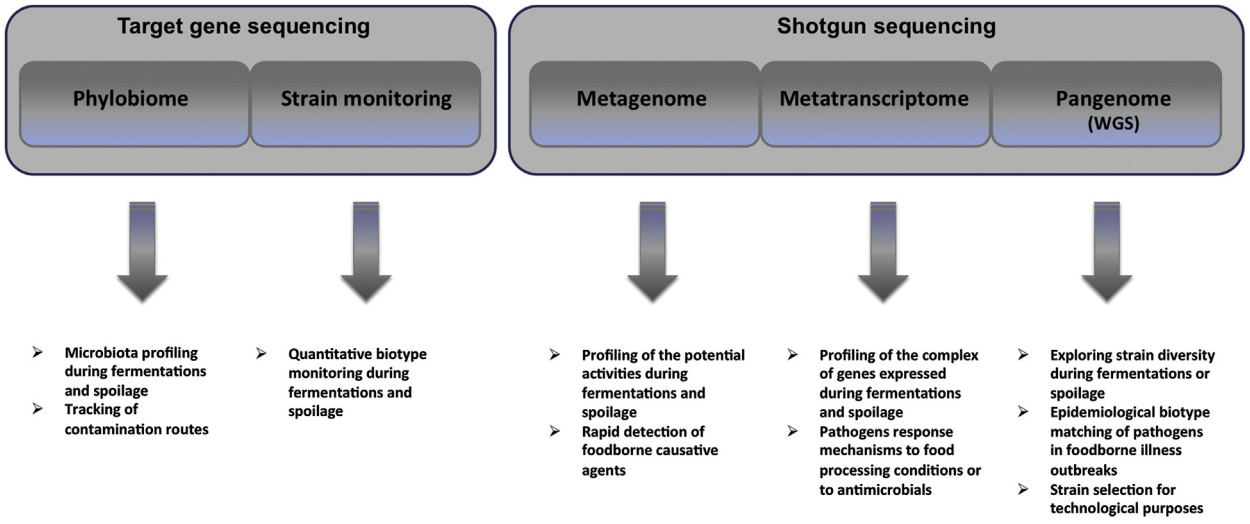

Figure 1 Possible applications of high-throughput sequencing (HTS) in food microbial ecology.

(OTU) is proportional to the abundance of that OTU in the sample sequenced, therefore we can estimate the occurrence (%) of different OTUs (Ercolini, 2013).

HTS Applications in Food Microbial Ecology Studies

Exploring the Microbiota during Food Production and Spoilage

The study of the microbiota, or phylobiome (van Hijum et al., 2013), through single-gene amplicon sequencing is the most exploited HTS application in food microbial ecology. The great diffusion of this application led to the development of many bioinformatics tools for data analysis collecting a number of stand-alone packages specific for each step in standardized pipelines, such as QIIME (Caporaso et al., 2010) or Mothur (Schloss et al., 2009), in order to meet the needs of microbial ecologists firstly approaching to this kind of analysis. The targets are genes of taxonomic interest, with the 16S rRNA being the gold standard for bacteria, also thanks to the availability of well-curated databases (McDonald et al., 2012; Cole et al., 2003; Pruesse et al., 2007). As for fungi, the most used target is the internal transcribed spacer (Adams et al., 2013; Bokulich and Mills, 2013a; Bokulich et al., 2013, 2014a; O'Sullivan et al., 2015). However, the uneven ITS length among species may promote preferential amplification (Bokulich and Mills, 2013a) and incorrect estimation of fungal OTU abundance. Therefore, the use of different targets is desirable, as recently proposed for the study of the fungal population of milk kefir grains (Garofalo et al., 2015), where the D1–D2 domain of the 26S rRNA gene was sequenced.

HTS of 16S rRNA gene was extensively used to study the microbial consortia in different dairy ecosystems (Ercolini et al., 2012; De Filippis et al., 2014; De Pasquale et al., 2014a,b; Dolci et al., 2014; Quigley et al., 2012a; O'Sullivan et al., 2015). The high sensitivity of HTS allowed detecting subpopulations previously not associated with cheese (Quigley et al., 2012a) and describing the complex microbiota of raw milk (Dolci et al., 2014; McInnis et al., 2015; Quigley et al., 2013). The microbiota during different cheese manufacturing was also deeply investigated. Water buffalo mozzarella cheese and intermediates of production were found to be dominated by thermophilic lactic acid bacteria (LAB) added through the natural whey culture (NWC) (Ercolini et al., 2012). On the contrary, mesophilic nonstarter lactobacilli evolved during ripening in the core of a medium-ripened pasta filata cheese and their abundance was positively correlated with free amino acid and several volatile compound concentrations (De Pasquale et al., 2014b). Recently, the microbiota of a continental-type cheese during ripening was found to be more complex in cheeses produced late in the evening compared to their early day equivalents (O'Sullivan et al., 2015), demonstrating the influence of the time of manufacturing within the same production day. HTS was also used to study fermentation processes of meat (Połka et al., 2015), olives (Cocolin et al., 2013), soybeans (Jung et al., 2014), kimchi (Park et al., 2012), and sourdough (Ercolini et al., 2013; Rizzello et al., 2015). Ercolini et al. (2013) showed that three types of flours had a really complex microbiota and share only few OTUs. Nevertheless, as soon as the water was added, a selected core microbiota, shared by the different sourdoughs, outcompeted other microbes, carrying on the fermentation. HTS studies of food spoilage microbiota were also carried out (De Filippis et al., 2013; Chaillou et al., 2015; Pothakos et al., 2014; Oakley et al., 2013). Psychrotrophic LAB were found as main spoilers of packaged and chilled-stored food products in Northern Europe (Pothakos et al., 2014). Moreover, in a recent study, the microbiota involved in meat and seafood products spoilage was deeply investigated (Chaillou et al., 2015), identifying sample type-specific OTUs in fresh samples. However, the spoiled samples shared a common core microbiota, mainly characterized by pshychrotrophic OTUs, highlighting the importance of low temperature in exerting a selective pressure. Indeed, also different packaging conditions may select a specific spoilage microbiota in beef (Ercolini et al., 2011). HTS approach was also employed for tracking back the sources of food contamination, since food processing plants and equipment are an important reservoir of microbes, that can be transferred to the product during manufacturing and processing and may act as spoilers or be beneficially involved in the productive process. Cheese (Bokulich and Mills, 2013b), beer (Bokulich et al., 2012), and sake (Bokulich et al., 2014a) fermentations were found to be driven by a beneficial plant resident microbiota. Moreover, the microbial populations on wine grapes were found to be influenced by season, as well as cultivar and vineyard location (Bokulich et al., 2014b), highlighting the presence of a regional specific microbial terroir and opening interesting clues on the possibility of a microbiota-based tracking of regional grapes. On the contrary, food processing environment can also be a source of spoilage microbes as demonstrated for beef (De Filippis et al., 2013), beer (Bokulich et al., 2015), and ready-to-eat meals (Pothakos et al., 2015). Finally, also residential kitchen and foodservice plant surfaces were investigated and found to be contaminated by bacteria associated with the skin, animals, and foods (Flores et al., 2013; Stellato et al., 2015). A challenging HTS application is the monitoring of microbes beyond the species level (Ercolini, 2013), since many phenotypic traits associated with spoilage (Ercolini et al., 2010; Casaburi et al., 2011, 2014) or positively affecting food production (Vaningelgem et al., 2004; Mora et al., 2002; Zago et al., 2012; Gori et al., 2012) are often strain-specific. Amplicon sequencing of target genes showing high sequence heterogeneity within a species may allow a quantitative monitoring of biotypes during fermentation or spoilage processes. In the only report about this application, *lacS* gene amplicon sequencing was used to monitor *Streptococcus thermophilus* beyond the species during curd fermentation of different cheeses (De Filippis et al., 2014). Therefore, genomic databases should be screened for the selection of possible target genes to be used for this promising application (Ercolini, 2013). The availability of sequences arising from studies of microbial ecology of foods and food-related environments opens the way to the interesting possibility of integrating all these different studies in meta-analyses. Foodmicrobionet (http://www2.unibas.it/parente/FMBN062web/) is a new tool recently developed (Parente et al., 2015) collecting data from multiple studies in an OTU-food samples network and allowing an easy and visual-effective comparison of one's own samples with several others belonging to the same food environment.

Whole Genome Sequencing to Understand the Genomic Potential of Food-Related Bacteria

HTS can be applied to the study of pure cultures, in order to improve our knowledge of pathogens or microorganisms involved in food chain (**Figure 1**). An in-depth knowledge of LAB genomics is crucial in order to understand their adaptation mechanisms to the specific food niches (Douillard and de Vos, 2014). For example, analysis of *Lactobacillus sanfranciscensis* genome highlighted the presence of genomic features which give it the ability to outcompete other species during sourdough fermentation, such as the presence of biosynthetic pathways for glutamate, glutamine, aspartate, and asparagine, usually at low concentration in wheat (Vogel et al., 2011). In addition, two clustered regularly interspaced short palindrome repeats (CRISPR) were identified. CRISPR loci have been suggested to confer resistance against bacteriophage DNA intrusion (Bolotin et al., 2005; Mills et al., 2010) and may explain the genetic stability of the *L. sanfranciscensis* strain in the sourdough propagation (Vogel et al., 2011). Nowadays, the rapidly reducing costs for genome sequencing allow to sequence multiple strains of the same species for comparative purposes, leading to the pangenomics, which is the pool of essentially different genes found within a species (Tettelin et al., 2008). Pangenomics studies can be useful in order to select the best strains as starters or probiotics or to explore strain differences in spoiling microbes (Garrigues et al., 2013). Tailored combinations of strains could be used as starter in fermentation processes, providing enzymatic cascades leading to specific properties. Pangenome of *Lactobacillus paracasei* (Smokvina et al., 2013) and *Oenococcus oeni* (Borneman et al., 2012) highlighted genomic differences with a potential impact on the industrial performance of these species, including cell wall exopolysaccharide biosynthesis, sugar transport/utilization, and amino acid biosynthesis. Whole genome sequencing (WGS) and single nucleotide polymorphisms may be useful also for epidemiological typing of pathogens (Kao et al., 2014; Bergholz et al., 2014). Harris et al. (2010) showed a geographical diversification of 63 strains of *Staphylococcus aureus*, while Lewis et al. (2010) used WGS of *Acinetobacter baumannii* strains isolated during a hospital outbreak and undistinguishable through conventional typing techniques to discriminate between different epidemiological hypotheses and individuate the patient who was the source of the pathogen. For these reasons, the US Food and Drug Administration (FDA) promoted a program for WGS (http://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWGS) of microorganisms isolated during foodborne illness outbreaks, in order to implement a database of foodborne pathogens, named GenomeTrakr, that already collected more than 10 500 *Salmonella* spp. and 2700 *Listeria* spp. isolate genomes (April 2015). The aim is to link the genomic information to the geographical origin of the isolates for tracking down the food source of a pathogen during an outbreak.

Metagenomics and Metatranscriptomics to Unveil the Complex Pool of Genes of Food Microbial Consortia

An HTS application still underexploited in food microbial ecology is the use of shotgun metagenomics and metatranscriptomics for the study of the microbiome, that is, the microbiota and its potential (metagenomics) or actually expressed (metatranscriptomics) activities (**Figure 1**). Diffusion of these powerful techniques is still limited due to the higher cost compared to target-gene sequencing, but most of all to the higher complexity of the bioinformatics analysis. In fact, although many tools for data analysis have been developed (Langmead and Salzberg, 2012; Li and Durbin, 2009; Trapnell et al., 2012; Zerbino and Birney, 2008; Schulz et al., 2012; Li et al., 2009; and many others), a ready-to-use pipeline as that proposed for rRNA gene data analysis is still not available. Few pivotal studies of metagenomics applied to food environment exist. It was used to describe the microbiome of marinated and unmarinated broiler meat (Nieminen et al., 2012) and kimchi (Jung et al., 2011) and the virome of retail beef, pork, and chicken (Zhang et al., 2014). Recently, coupling pangenomics and metagenomics, Erkus et al. (2013) demonstrated the mechanisms assuring the maintaining of the strain diversity in a NWC during back-slopping: the phage-sensitivity of the fittest strain seemed to be density-dependent, avoiding the suppression of other genetic lineages. In another study, microbial consortia and functions of washed, natural, or bloomy cheese rinds were studied and many pathways leading to aromatic compounds were identified as enriched in washed rind cheeses (Wolfe et al., 2014). Application of metagenomics to foods may be promoted and enhanced by the availability of curated gene catalogs specific for each environment, as it happened for gut metagenomics (Qin et al., 2010). Therefore, research projects such as that by Almeida et al. (2014), who implemented the first dairy microbial genome catalog, are of invaluable importance. Although DNA-based metagenomics can provide important information, when DNA is the target of our analysis, we can only talk about potential activities, since it may arise from dead or metabolically inactive cells. If we want to study the pool of genes actually expressed during the manufacturing or spoilage of a food product, we have to study its metatranscriptome through RNA sequencing (RNA-seq), as already done for kimchi (Jung et al., 2013). RNA-seq is intended to replace microarrays in HTS metatranscriptomics studies that, although revealing valuable information, relies on probe design, limiting the biodiversity that can be detected (van Hijum et al., 2013). Only few metatranscriptome studies in food environment are available. Lessard et al. (2014) studied through RNA-seq the gene expression of *Penicillium camemberti* and *Geotrichum candidum* during ripening of a Camembert-type cheese and identified many pathways leading to flavor compounds production, while recently Dugat-Bony et al. (2015) combined metagenomics, metatranscriptomics, and biochemical analyses to monitor microbial activities during the ripening of a small-scale surface-ripened cheese inoculated with nine selected strains. Metagenomics and metatranscriptomics studies are of invaluable importance in order to understand the complex mechanisms involved in food fermentations and spoilage. Moreover, metagenomics has been proposed as a powerful tool also for a rapid detection of the causative agent of a foodborne disease (Bergholz et al., 2014), as already proposed for outbreaks of *Campylobacter jejuni* (Nakamura et al., 2008) and Shiga-toxigenic *Escherichia coli* O104:H4 (Loman et al., 2013). For this purpose, a new bioinformatics tool able to infer strain-level identification from metagenomics data has been recently developed (Ahn et al., 2015). Also in this case, it has to be pointed out that the use of DNA does not assure that the microorganisms are still alive. In addition, the study of the metatranscriptome

of foodborne pathogens can unveil the mechanisms involved in their response to common antimicrobial agents (Visvalingam et al., 2013; Casey et al., 2014) or to the conditions normally used during food processing and storage (Goudeau et al., 2013; Fink et al., 2012), helping to understand how to kill them or how to prevent their growth.

Critical Issues

HTS is uniquely quantitative. The number of sequence assigned to an OTU or to a gene is proportional to its abundance in the sample. This allows comparing the profiles of more samples. However, also HTS suffers from biases due to sample handling (Brehm-Stecher et al., 2009) or nucleic acid extraction (Quigley et al., 2012b; Cruaud et al., 2014; Guo and Zhang, 2013; McCarthy et al., 2015) that have to be carried out avoiding any possibility of alteration of the original microbiome. In fact, different cellular wall organization may lead to preferential extraction of nucleic acids from some species at the expense of others. In addition, when messenger RNA (mRNA) is the target of a metatranscriptomics analysis, precautions have to be taken in order to 'freeze' the gene expression in the moment of the sampling, since microbial mRNA has extremely short half-life (Rauhut and Klug, 1999; Deutscher, 2006). For this purpose, solutions are available on the market that promise to protect RNA from degradation, such as RNAlater (Ambion) or RNAstable (Biomatrica). Another possible source of bias is the possibility of preferential amplification, when a PCR step is present (Sipos et al., 2010; Pan et al., 2014; Pinto and Raskin, 2014). Therefore, primer design has to be done carefully and taxonomic binning of shotgun metagenomics reads can be considered more reliable since avoiding biases arising from PCR (Liu et al., 2011). Moreover, it has to be considered that food fermentation and spoilage dynamics often involve the succession of different species of the same genus, requiring a taxonomic assignment as deep as possible. Nowadays, rRNA gene studies of the food microbiota can benefit the sequencing of longer reads including more variable regions for a deeper and more reliable taxonomic assignment. Sequencer actually available on the market can reach 800 bp, while new technologies such as those developed by Pacific Biosciences, and more recently Oxford Nanopore, promise even longer reads.

Another important issue to consider carefully is the data analysis pipeline to follow, as every step from sequence quality filtering up to taxa or gene assignment can influence the results obtained. A critical point is the choice of a well-cured and up-to-date database, which is still an issue when considering fungi, since available databases are considered less curated than bacterial ones or with a lower coverage (Nilsson et al., 2006; Tedersoo et al., 2011). The most complete database for fungi collects ITS sequences, that, as stated above, is often not the best choice because of the possibility of preferential amplification due to the uneven length of the ITS fragment in the different fungi species. Finally, important points to consider are the necessity of skilled bioinformaticians and large computational resources that can be reckoned the real bottleneck in HTS studies of microbial ecology, in particular in shotgun metagenomics or metatranscriptomics. In order to overcome these limitations, a new tool has been developed (http://picrust.github.io/picrust/, Langille et al., 2013) promising to predict the metagenome starting from 16S rRNA gene for bacteria, while fungi are not supported yet. It will surely benefit the growing availability of sequenced and annotated genomes and maybe in the future we will be able to predict the potential activities of the microbiota just with the easy sequencing of a marker gene.

Conclusions

We actually have all the tools to deeply understand the microbial world. HTS tools are rapidly changing our approach to the study of microbial ecology in food ecosystems and offer an invaluable opportunity to shed light on microbial dynamics involved in food production and spoilage. Nevertheless, the limitations of omics application rely on the relatively high cost and, most of all, the need of specific bioinformatics skills for data analysis, that still restrain the escalation of these approaches to the industry.

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