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Current perspectives in food-based studies exploiting multi-omics approaches

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The new frontiers of microbial ecology are concerned pertain to what microbes are do in a complex ecosystem, such as food, and how the environmental conditions (*e.g.*, changes in the process parameters, storage temperature, the addition of a starter culture and changes in ingredients) can affect the development and functioning of microbiota. A multi-omics approach can help researchers to obtain an unprecedented insight into the mechanisms that can affect the final characteristics of products, in term of organoleptic proprieties, as well as safety.

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Introduction

Next-generation sequencing and metagenomics were first used in microbial ecology in the second decade of the 2000s. At present, a search on the ISI Web of Knowledge on the topics 'metagenomics' and 'food' shows the presence of 660 research papers, with less than 90 per year before 2013, a peak of 132 in 2015 and 109 in the first 10 months of 2016. This exponential increase in studies is due to the greater availability of sequencing centers with competitive prices, along with a growing population of scientists with a good background in bioinformatics and biostatistics, as well as the development of online platforms that allow a huge amount of data to be analyzed, even by inexperienced researchers. The term metagenomics is a miscellaneous term that is often misused by many researchers. Metagenomics is the appropriate term for a shotgun approach in which all the genome contents from the matrix are sequenced (host, gene fragments of taxonomic interest, as well as functional genes); instead, if a taxonomic region is massively sequenced (16S, ITS or 26S), the term that should be used is amplicon-based sequencing. The first decision that a researcher has to make is whether to adopt global or live high throughput sequencing (HTS). This is the crucial issue that has to be resolved before starting an experiment, since the use of DNA or rRNA as targets can lead to both advantages and disadvantages. DNA is more stable and easier to extract and manipulate, but a DNA experiment displays the global microbial population, including DNA from dead and damaged cells, as well as from live cells, with the consequence that a researcher will not be able to discern whether the microbiota is still alive and active or dead at a specific sampling point. The decision to use RNA as a target eliminates this bias, because RNA, after cell lysis, is less stable than DNA, and allows the analysis to be focused only on live and active microbiota [1]. On the other hand, the disadvantage of using rRNA as a target is the amplification of ribosomal genes, due to the operon copy number, which varies widely across the taxa, and can even distort the quantitative diversity estimates [2]. Another possible way of detecting live populations is through the use of the DNA of ethidium monoazide (EMA) and propidium monoazide (PMA), which can prevent the amplification of DNA from dead cells.

Increased data analysis skills can allow the study of microbial composition (amplicon target sequencing), gene content (meta-genomics), gene function (meta-transcriptomics), functional activity (meta-proteomics) and metabolites (meta-metabolomics) to be joined together. The huge amount of data generated through a multi-omics approach can improve the knowledge on what really happens in a complex process, such as in the food fermentation process, or in general during a process that involves microbes.

High-throughput amplicon target sequencing

The first and most frequently applied HTS technique is the application of amplicon target sequencing to the microbial composition of a food matrix in order to study the microbiota (targeting the 16S gene) or the mycobiome (targeting the ITS or the 26S gene) of the food. The flurry of research has been witnessed over the past couple of years aimed at estimating the microbial diversity in different dairy ecosystems using 16S DNA as the target. Several studies on food have clearly shown the presence of several contaminant taxa, probably originating from the environment, which can play a role in the decay of food quality. However, the main objective of all of these studies has been to assess the microbial structure of

the analyzed product in order to find a correlation between the external perturbations (*e.g.*, changes in the process, ingredients and sampling point) and the evolution of the microbial composition. Table 1 reports an extensive, although not complete, list of these studies.

In the targeted amplicon technique, the most common approach adopted to study the mycobiome is that of amplifying the fungal 'internal transcribed spacer' (ITS) regions. Since these ITS regions are not part of the conserved transcribed regions of the structural ribosomal RNAs, they are highly divergent between fungi, and are often sufficiently different to allow the fungi to be classified at species level. The locus in fungi is generally duplicated 100–200 times, thus caution must be used when trying to derive quantitative comparisons between various species in mixed populations through this approach. First, unlike bacterial 16S amplicons, fungal ITS sequences from different species can differ to a great extent in size and sequence content [28]. ITS fragments generally vary in length from between 100 and 550 base pairs, and it is not yet clear how the variable lengths affect the recovery of sequences through the various steps of sequencing on high-throughput platforms. In addition, there is no well-established database of ITS sequences. The publicly available repositories of fungal sequences are replete with redundant sequences containing incomplete and/or incorrect taxonomic assignments [29]. Most fungi show high inter-specific variability in the variable D1/D2 domain of large subunit (26S) ribosomal DNA [30], and sequencing appears most robust because strain comparisons can easily be made. Recent studies [11•,29–32] have indicated that the use of the D1/D2 region of the 26S rRNA gene, using NL1 primers to investigate the fungal distribution in the samples, appears to be the most robust approach. However, more work still needs to be done to implement and make a database, such as Greengenes, available for 16S.

Only a few papers have been aimed at understanding what the microbiota really does in a food matrix by coupling HTS with other techniques, thus representing complete and comprehensive studies. Interesting results have been obtained from these studies, and they clearly show that only a few taxa really play important roles during the food process, and that it is only by coupling different techniques that it is possible to study complex food ecosystems. In addition, one of the important questions that need to be addressed, once the microbiota composition has been evaluated, is how this microbiota (in most cases a few taxa) can affect the final characteristics of the products. One possible approach is to couple

Table 1

Amplicon target sequencing studies on different food matrices

Target	Short description	Food matrix	Reference
16S DNA	Bacterial diversity of Salame Piacentino PDO during ripening	Meat	[3]
16S RNA (cDNA)	Piedmontese fermented meat during ripening	Meat	[4]
16S RNA (cDNA)	Beef burger (controls or with added preservatives, nisin + EDTA) vacuum packed	Meat	[5]
16S DNA	Vacuum-packaged, cooked sausage	Meat	[6]
16S DNA	Fresh beef and pork cuts	Meat	[7]
16S DNA	Fresh and spoiled meat and seafood samples	Meat/fish	[8]
16S DNA	Chicha, a maize-based fermented beverage from Argentina	Fermented beverages	[9]
16S DNA	French organic sourdoughs	Doughs	[10]
16S RNA (cDNA)/16S DNA	Olive surfaces and brine during spontaneous and inoculated fermentation	Vegetables	[11•]
16S RNA (cDNA)	Wheat flour grown under organic and conventional farming conditions	Doughs	[12•]
16S DNA/26S DNA	Milk kefir grains collected in different Italian regions	Fermented beverages	[13]
16S DNA/ITS DNA	Samples from spontaneous 'Vino Santo Trentino' fermentation	Fermented beverages	[14]
16S DNA	Microbiota of Belgian white pudding after refrigerate storage	Meat	[15]
16S DNA	Rind and core microbiota of Caciotta and Caciocavallo cheese	Dairy and fermented milks	[16]
16S DNA	Mozzarella cheese made from cow's milk and produced with different acidification methods	Dairy and fermented milks	[17]
16S DNA/18S DNA	Naturally fermented cow's milk collected from Mongol-ethnic families	Dairy and fermented milks	[18]
16S DNA	Pico cheese made from raw cow milk	Dairy and fermented milks	[19]
16S DNA	Spoiled hard cheeses during ripening	Dairy and fermented milks	[20]
16S DNA	Brine-salted continental-type cheese	Dairy and fermented milks	[21]
16S DNA	Poro cheeses manufactured with different milk	Dairy and fermented milks	[22]
16S DNA	Herve cheeses from both raw and pasteurized milk	Dairy and fermented milks	[23]
16S RNA (cDNA)	Piedmont hard cheese made from raw milk: milk, curd and cheese throughout ripening	Dairy and fermented milks	[24]
16S RNA (cDNA)	Milk, curd and Caciocavallo cheese during ripening	Dairy and fermented milks	[25•]
16S RNA (cDNA)	Milk (from different lactation stages), curd and Fontina cheese from three different dairies	Dairy and fermented milks	[26]
16S DNA/18S DNA	Fermentation of Pu-erh tea	Fermented beverages	[27••]

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