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

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Abstract

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**Abstract**

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.

**Highlights**

- Bioinformatics tools have been developed to provide information on microbe diversity
- Shotgun metagenomics is a promising approach to discover the functions of microbiota
- Data generated through a multi-omics approach can improve the knowledge on what happens in food

## 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.

53 Increased data analysis skills can allow the study of microbial composition (amplicon target
54 sequencing), gene content (meta-genomics), gene function (meta-transcriptomics), functional
55 activity (meta-proteomics) and metabolites (meta-metabolomics) to be joined together. The huge
56 amount of data generated through a multi-omics approach can improve the knowledge on what
57 really happens in a complex process, such as in the food fermentation process, or in general during
58 a process that involves microbes.
59
60 **High-throughput amplicon target sequencing.**
61 The first and most frequently applied HTS technique is the application of amplicon target
62 sequencing to the microbial composition of a food matrix in order to study the microbiota (targeting
63 the 16S gene) or the mycobiome (targeting the ITS or the 26S gene) of the food. The flurry of
64 research has been witnessed over the past couple of years aimed at estimating the microbial
65 diversity in different dairy ecosystems using 16S DNA as the target. Several studies on food have
66 clearly shown the presence of several contaminant taxa, probably originating from the environment,
67 which can play a role in the decay of food quality. However, the main objective of all of these
68 studies has been to assess the microbial structure of the analyzed product in order to find a
69 correlation between the external perturbations (e.g. changes in the process, ingredients and
70 sampling point) and the evolution of the microbial composition. Table 1 reports an extensive,
71 although not complete, list of these studies.
72 In the targeted amplicon technique, the most common approach adopted to study the mycobiome is
73 that of amplifying the fungal "internal transcribed spacer" (ITS) regions. Since these ITS regions
74 are not part of the conserved transcribed regions of the structural ribosomal RNAs, they are highly
75 divergent between fungi, and are often sufficiently different to allow the fungi to be classified at
76 species level. The locus in fungi is generally duplicated 100–200 times, thus caution must be used
77 when trying to derive quantitative comparisons between various species in mixed populations
78 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 interspecific 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 the HTS-amplicon based approach with metabolomics (both targeted and untargeted) to create a tool that can be used to identify the potential candidate metabolites (biomarkers) related to specific taxa [33].

**Bioinformatic tools to translate sequences into data for interpretation purposes**

Recently, several tools have been developed to use the data from amplicon base sequencing as input and to analyze these data so as to provide information on the diversity of the microbes. Network

105 analysis [34••] has emerged as an important tool that can be used to easily observe the structure and
106 dynamics of microbes, from an interactive point of view of the microbiota distribution, which can
107 also be used for food process development. Gephi or Cytoscape software can help scientists to
108 visualize data and to easily extract information about the development or the interaction of the
109 microbiota in the samples. Foodmicrobionet (http://www.foodmicrobionet.org/fmbn1_0_3web/) is a
110 recently developed application that collects data from multiple food-based studies with the aim of
111 allowing an easy and visual-effective comparison of one's own samples with several others from
112 the same food environment [34••].
113 Amplicon-based sequencing is a key tool for studies on microbial communities, but does not
114 provide direct evidence on a community's functional capabilities. An easy way of getting an idea of
115 the potential function of the microbial community is to use a computational approach to predict the
116 functional composition of a metagenome, using marker gene data and a database of reference
117 genomes. PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved
118 states) shows that the phylogenetic information contained in 16S marker gene sequences is
119 sufficiently well correlated to the genomic content to provide an accurate prediction of the gene
120 repertoires, associated with their microbiota [35]. The main application of this tool is to
121 environmental samples, however, in food associated studies, the tool has been found to be able to
122 find correlations among taxa and metabolic functions associated with spoilage [5,7].
123 Another promising NGS data analysis method relies on the use of oligotyping, a novel supervised
124 computational method that can elucidate concealed diversity from within the final operational units
125 of classification or clustering approaches. Unlike clustering methods, which compare all the
126 positions in sequence reads to assess similarity, oligotyping utilizes the nucleotide positions that
127 have been identified as the most information-rich, and allows resolution at a species level or even
128 below [36]. Till now, only human-based and environmental studies have used this tool to identify
129 sub-OTU level differences across samples [37], or to track changes in specific populations across
130 seasons and geography [38]. However, this tool can also be easily applied to food based studies in

order to ascertain an association between an oligotype and a process, or to have a better idea of the distribution of a specific taxon in a food-based system.

**Who is there and what are they doing?**

The shotgun metagenomic approach (DNA-seq or RNA-seq) is a valuable approach that is applied extensively to environmental microbiology, but which is also of increasing interest in food microbiology. The main purpose of this technique is to obtain, at the same time, information about the microbe composition and the gene content without any PCR bias. Interest in the shotgun RNA-seq approach, applied to food matrix, is growing, due to its ability to discover the functions of microbes during a food process. This technique has recently been applied to cheese matrices in order to find differences in gene expression associated with a particular ripening time [39], to select biological markers in order to improve cheese quality assessment [40], or just to assess the microbial physiology during cheese manufacturing [41,42]. The main problem of using RNA-seq alone is the lack of availability of genome sequences to map the reads, and the need to couple them to DNA-seq data and to the amplicon-based HTS data, which results in an increase in the cost of sequencing. The use of the shotgun DNA-seq approach is interesting, because it provides higher-resolution taxonomic information than 16S rRNA sequencing and can profile hundreds of uncharacterized species, especially those present in low abundances, and at the same time obtain information about the gene content from a global point of view. The main application in food concerns the possibility of detecting foodborne pathogens in a food matrix [43,44], or of understanding the change in the gene content during a process [45-48]. A possible application of DNA-seq concerns the possibility of performing a de novo extraction of strains from metagenomes. Pangenome [49] is used extensively in epidemiology studies with the aim of analyzing strain-specific gene sets, and of providing a comprehensive view of the functional and pathogenic potential of the organisms. When reference genomes are included in the analysis, it is also possible to compare different strains or to identify new ones. This tool is promising for food ecologists, and

157 can easily be applied to food systems in a variety of ways, such as the selection of species/strains
158 for starter cultures, or the discovery of possible associations between a specific strain and a process
159 point. The increase in scientists' bioinformatic skills, the availability of online tools to analyze data
160 (e.g. MG-RAST, Galaxy) and the increase in the number of pipeline applications, such as
161 PanPhlAn [50] or Anvio's [51], all allow the huge amount of data produced with/through the
162 shotgun metagenomic approach to be analyzed.
163
164 **Multi-Omics Approach**
165 Most of the studies based on NGS just give a partial representation of the food-based ecosystem,
166 because only one of the techniques is applied, and a final remark, such as "…needs further study
167 …", is often added. In the authors' opinion, this is probably due to the cost of the experiment or the
168 need for different specialties, which are generally lacking in a single research unit. Only a few
169 examples that combine different omics approaches have been found for food. Dugat-Bony et al.
170 have recently shown an example in which data from metagenomic, metatranscriptomic and
171 biochemical analyses have been combined to obtain a complete view of what really happens during
172 the process [42••]. De Filippis et al. [39••] have also clearly shown that coupling
173 metatranscriptomic and metabolome data is effective in discovering the functional diversity of
174 cheese microbiota affected by different ripening conditions. Coupling the genetic potential and
175 final phenotype to, for example, metabolomics and metaproteomics, which is also called
176 proteogenomics [52], can offer the possibility of resolving the main functional components that
177 drive the function of the microbial ecosystem [53]. Proteogenomics can in particular offer the
178 possibility of exploring the microbial function, although metagenomics analysis can detect the
179 presence of different bacterial species and genes, metaproteomics can/is able to provide information
180 on the most representative metabolic pathways that are active during the food process [54].
181
182

## Conclusion

At the moment, several tools are available to help one really understand what happens in a food-based system. Unfortunately, only a few examples of multi-omics approaches are available in the literature and these approaches need to be implemented to obtain a better understanding of food microbial ecosystems. However, this approach also suffers from certain limitations, due to its relatively high cost and the need for specific bioinformatics and biostatistics skills for the data analysis.

191 **Table 1** Amplicon target sequencing studies on different food matrices
192

| Target | Short description | Food matrix | Referen |
|---|---|---|---|
| 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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196 • of special interest

197 •• of outstanding interest

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