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Zooming into food-associated microbial consortia: a "cultural" evolution

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Abstract

Foods are complex and dynamic microbial consortia where bacteria, yeasts and filamentous fungi can coexist. The advances in the culture-independent analysis of food microbiota have completely revolutionized the way we study these microbial ecosystems, leading to a "cultural" evolution. This is not only because we have technically learned to avoid cultivation to study food microbes, but also because our mental approach to food microbiology has changed. In this article we discuss the most recent achievements in the field of food microbial ecology and give examples of how current molecular biology tools can be profitably used to study performance of microorganisms used for food fermentation, to explore the sources of technologically relevant and spoilage bacteria as well as to acquire fundamental knowledge on behaviour of foodborne pathogens. Although we will still be in need for cultivation for several reasons, we currently have an amazing toolbox that will enhance the chances to gain fundamental insights from the food microbiome studies that will further change the overall approach to food science.

Introduction

Regardless of their complexity, microbial populations in foods are the object of one of the widest attention from the scientific community because of the great influence they can have on quality and safety issues that are pivotal in food science. Foods are complex and dynamic microbial ecosystems in which bacteria, yeasts and filamentous fungi are cohabiting, interacting and communicating. Through these activities they contribute to the transformation of raw materials in final products in the case of food fermentations, but they can be also responsible for food spoilage. The advance in the technologies of analysis of food microbiota have completely revolutionized the way we study these microbial ecosystems and have allowed us to make progress in leaps and bounds in food microbial ecology. Food microbiologists often report in their scientific articles that the bias of cultivation can be overcome by the use of culture-independent approaches. At present, we are not yet fully sure that culturability is as much an issue in food studies as it is in other natural environments. Indeed, recent studies demonstrated the possibility of a full *in vitro* reproduction of complex microbial communities from cheese [1**]. Food microbial ecology has been based on the study of microbial isolates for decades. Still, culture-independent methods have allowed for the last 20 years a convenient and reliable analysis of food microbiota for the most different purposes. It all started with the translocation of the polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) approach from the field of environmental microbiology, where it was firstly used [2], to the analysis of food samples, where it was employed to monitor microbial populations during food production, storage and distribution to look at food fermentation and spoilage dynamics [3,4].

With the advent of the culture-independent analysis of food, it is a "cultural" evolution we have been experiencing. This is not only because we have technically learned to avoid cultivation to study food microbes, but also because our mental approach to food microbiology issues has changed. We have evolved to think of food microbes as consortia and learned to monitor their occurrence, changes and activities as such.

The structure of food microbiota described by a bunch of sequences

Recently we have switched from fingerprinting analysis in which food microbiota is visualized as band patterns, to sequencing where microbial consortia are described as nucleic acid sequences (Fig. 1).

The current availability of the most powerful high-throughput sequencing (HTS) technologies have determined an almost complete replacement of the electrophoretic PCR-DGGE approach with direct sequencing of the rRNA genes of mixtures of barcoded food samples. At the moment, the novel sequencing-based tool is massively used in many research laboratories active in food sciences and it is pushing forward the culture-independent study of food microbial ecology [5]. The use of rRNA amplicon sequencing to study microbiota is the most common HTS application in food microbial ecology, although cost of analysis and need for bioinformatics skills are limiting industrial applications [5]. This entails the analysis of amplicons arising from a complex mix of microbial genomes directly extracted from a food sample. The target genes are those of taxonomic interest, with the 16S rRNA gene being the most widely used for bacteria, while ITS and 18S for fungi. rRNA amplicons, obtained from DNA/RNA extracted directly from foods, are sequenced and sequences are compared to reference databases to identify the operational taxonomic units (OTUs) through well established bioinformatics pipelines [6-8]. The approach, recently re-named metagenetics [9], is considered quantitative as the number of sequence reads identified with the same OTU allows for an estimation of the relative abundance of each microbial entity in the food sample analysed. The methods require a constant and careful updating and maintenance of the sequence databases that are almost weekly enriched with new sequences. The unprecedented advantage of sequencing-based tools is having a quantitative monitoring of "microbial species" in food ecosystems. The quantitative power of the PCR-based target amplicon sequencing is widely acknowledged by the scientific community. However, we would like to take this opportunity to remind that such tools bring all the possible bias linked to potential selective nucleic acids extraction and preferential PCR amplifications [5] that are currently perhaps too much overlooked. With sequencing-based detection systems, the achievement of species-level taxonomic identification can be very tricky and strongly depends on the sequences length. In several food products, varying from cheese to meat or fermented vegetables, many different species of the same genus can occur. In such cases, an HTS study at

the genus level is not useful as being informed that "*Lactobacillus*" dominates in a cheese ripening is no news. For this purpose, long sequence reads including more variable regions of the 16S rRNA gene are required for accurate assignment.

Studying the changes in microbial populations can 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. Many different researches have been carried out unraveling the structure of the microbial consortia in dairy [10-14], meat [15] and vegetable foods [16*-19].

Food fermentations can be observed at community level with a glance at microbe-samples networks. In a case of sourdough fermentation, sourdough-microbe networks based on RNA data obtained by pyrosequencing of 16S rRNA gene amplicons show the microbial evolution in sourdoughs (Fig. 2). Three different cereal flours have very complex microbial communities and do not share much in terms of common OTUs. However, as soon as water is added to start the fermentation, the microbial diversity decreases and the number of shared microbes increases as the propagation proceeds, making the three final sourdoughs very similar [20].

Exploring the sources of food contamination is also a very hot and challenging topic in food microbial ecology and the assessment of the microbial communities in the food-related environments can be crucial. This can be important to recognize the sources of microorganisms important for the fermentation of cheese [21, 22], beer [23] and wine [24] or to understand where food spoilers can come from [25, 26]. Very often raw materials are the main source of important microbial players in premium-quality fermentations and they frequently have specific traits that are conditioned by the geographical origin [27]. Recently, in a research project describing the microbial biogeography of wine grapes we had a proven evidence of how vintage and climatic, regional, site-specific, and grape variety factors can shape the fungal and bacterial consortia occurring on wine-grape surfaces and how these factors determine the unique microbial inputs to regional wine, highlighting the existence of a microbial *terroir* as pivotal in regional variation among wine grapes [28**].

We are also very interested in detection and monitoring of microbes beyond the species-level identification. Strain typing is of great importance for in-depth investigation of microbial dynamics in foods. Culture-independent strain monitoring is crucial to assess the performance and fitness of starter cultures as well as to investigate the role of specific strains in food spoilage. It would be desirable that all the whole genome sequences that are piling up in the public databases could be used to detect intra-species sequence heterogeneities to develop sequencing-based protocols for a quantitative monitoring of different biotypes of the same species in foods. Such opportunity has been recently exploited to look at diversity of *S. thermophilus* population in different cheeses [29*].

Overall, the sequencing-based culture-independent approach to food microbial ecology is not only faster and more reliable than culture-based microbiology. Given its sensitivity, it offers the unprecedented opportunity to profile sub-dominant microbial populations thus contributing to actually "zoom" into microbial consortia in order to better describe the key microbial players and their dynamics. Moreover, with the progress and optimization of "custom" bioinformatics pipelines, it will be possible to link the structure of microbiota and the relative abundance of microorganisms to any other measurable variable in the food ecosystem such as abundance of metabolites, changes in food structure etc., which offers valuable cues to infer the role of specific microbes in foods.

Tantalizing opportunities to zoom into microbial functions in food

Surely we do not only aim at defining the structure of the microbiota and addressing the question of 'who is there', we evolved timely enough to turn and look at the expression of microbial activities directly in food. Such opportunity is given by metagenomic, metatranscriptomic and metaproteomic studies that are intended to study the food microbiome, i.e. the microbiota with all its potential functions. These are carried out by starting with DNA, RNA and proteins directly extracted from food, respectively.

The meta-omics offer tremendous chances to look at fermentation and ripening dynamics in ripened foods through the analysis of the genes expressed during such events and how technological parameters (temperature, humidity, ingredients etc.) employed by the food industry may affect, and may be changed to affect, the microbiome and its activities. Pioneer studies in metagenomics have been carried out to look at changes in the potential microbial gene functions during storage of marinated and unmarinated broiler meat [30], to describe the metagenome of kimchi [31] and also to look at viral communities in fermented foods [32]. Metagenomics allows testing important ecological hypotheses. In a dairy starter culture of defined composition, the degree of biodiversity at strain level is probably maintained by a density-dependent phage sensitivity of the fittest strains that prevents the complete eradication of some genetic lineages from the starter culture during propagation [33*].

As recently demonstrated [1**], some food microbiomes can be excellent models to investigate on the mechanisms regulating the assembly of microbial communities in natural environments. Microbiome in cheese rinds is a good example of biofilm formation, its development is affected by technological parameters more than geographical regions of cheese origin, and metagenomics indicates that cold-adapted proteolytic and lipolytic enzymes could effectively contribute to cheese ripening [1**]. Recently, a metatranscriptomics-based monitoring of fungal biological activities of the dominant ripening microflora of Camembert cheese contributed a solid basis to select biological markers to improve cheese quality assessment [34].

However, shotgun DNA- and RNA-seq are still relatively expensive and the data are not easy to deal with in order to have a reliable idea of the distributions of genes and functions in food. Interesting alternatives are given by powerful bioinformatics tool that can be used to predict the metagenome starting from the 16S-based structure of the microbiota [35] although this has not been used yet in food environments. It should be underline that, due to the robustness of the DNA molecule, metagenomic approaches are not able to monitor functions that are expressed when the analysis is performed and they can also result from dead microbial cells. In order to determine expressed functions from viable populations, metatranscriptomics should be employed.

Next generation studies of food safety

Microbial consortia may unfortunately include foodborne pathogens. Therefore, one of the reasons to zoom into food microbial populations is to address food safety issues.

Modern sequencing approaches have tremendous potentials in the field of food safety. Having the possibility to investigate the whole DNA and/or RNA extracted from a food matrix allows not only for a better understanding of the microbial ecology, but also for the comprehension of the prevalence and the behaviour of foodborne pathogens without the necessity to isolate them.

Through metagenomics, pathogens can be identified by searching at specific DNA signatures within the sequences obtained directly from a sample (either a food sample or a clinical sample). An example of such application is the recent study published by Loman et al [36**], who exploited metagenomics for an in depth investigation of the *Escherichia coli* O104:H4 outbreak occurred in Germany in 2011. Analysing 45 samples obtained from patient with diarrhoea, 27 of 40 Shiga toxin-producing *E. coli* (STEC)-positive samples (67%) contained specific sequences from the Shiga-toxin genes, moreover sequences from *Clostridium difficile, Campylobacter jejuni, Campylobacter concisus,* and *Salmonella enterica* were also recovered. The results of this study clearly highlight the potential of metagenomics for a culture-independent identification of bacterial pathogens in foods and for their tracking in cases of outbreaks. As indicated above, due to the lack of correlation between DNA presence and microbial vitality, the results coming from such approaches do not allow to understand if the pathogenic microorganism is alive or not.

The whole genome sequencing (WGS) can also provide answers to address public health problems. Franz et al. [37], in their review, analyse in detail how the blooming information associated with whole genome sequencing can be exploited to tackle STEC in global food production systems. Undoubtedly the WGS approach can provide useful information in order to better understand the evolution, virulence and epidemiology of STEC. For the food industry, the linking of genomics data to phenotypic response could lead to far more detailed and accurate quantitative microbiological risk assessment in foods, which will allow better control of

foodborne pathogens.

Understanding of microbial behaviour in foodborne pathogens is pivotal in food safety. This approach takes into consideration the expression of specific traits, namely virulence and stress responses, *in situ*. From studies conducted so far, it has been shown that within species of pathogenic bacteria, strain heterogeneity in terms of expression of virulence genes exists. Furthermore, environmental conditions that pathogenic microorganisms encounter in foods influence their stress response capabilities, enhance survival and possibly overall virulence potential [38].

The study of foodborne pathogens behaviour has been conducted in the last years through the application of reverse transcription quantitative PCR (RT-qPCR) and microarrays. Table 1 summarises the scientific contributions that have been published in the last five years. Taking into consideration the new information produced applying transcriptomics *in situ* and comparing them to the previous evidences collected in laboratory conditions, it becomes evident that some of the consolidated knowledge in foodborne pathogens physiology and regulation should be reconsidered since pathogen behaviour *in situ* seems to be different from *in vitro*.

In the context of NGS, metatrascriptomics will contribute in the comprehension of the mechanisms involved in virulence, stress response, adaptation and host-pathogen interaction. Unfortunately there are just few examples of exploitation of metatrascriptomics related to the food sector. Casey et al. [50] investigated the transcriptomic response of a persistent strain of *Listeria monocytogenes* on exposure to a sub-lethal concentration of the quaternary ammonium compound benzethonium chloride. Using RNA-Seq, hundreds of differentially expressed genes were identified, and suggesting that many biological processes such as peptidoglycan biosynthesis, bacterial chemotaxis and motility, and carbohydrate uptake, are involved in the response of *L. monocyotogenes* to the disinfectant.

Transcriptomic and metatranscriptomic data are extremely relevant when prediction of microbial history-dependent behaviour have to be determined. Interestingly, den Besten et al. [51] have investigated with microarrays the ability of *Bacillus cereus* to overcome mild stresses and induce enhanced microbial robustness towards lethal stresses. The approach proposed by the authors provides a framework to search for biomarkers and to evaluate their predictive quality in order to select those that can serve to early detect and predict adaptive traits.

We will still want to culture though!

In an evolved food microbiology context where culture-dependent are being substituted with culture-independent methods and researches are more willing to acquire bioinformatics skills rather than continuing to deal with Petri dishes and culture media, the cultivation and isolation of microorganisms is still deemed as strictly necessary in certain domains of investigation.

Scientists who deal with development of starter cultures for food fermentations will still need to isolate, characterize and maybe induce fitness traits in the possible strains to be used to start food fermentations. In addition, strain-dependent spoilage behaviour has been demonstrated in food spoilers. Therefore, food microbiologists will keep chasing spoilage-associated isolates in order to test their actual contribution to food spoilage, their potential interactions and their sensitivity to food and food storage conditions. Also in these contexts NGS is contributing to better understand the phenotype of the selected strains and is expected to bring new knowledge from genome and transcriptome investigations.

Conclusions

We have all the tools to adequately study microbial ecology in foods and the golden opportunity to use these tools to bridge the major gaps and address the most important issues in food microbiology. The approaches described in this paper will allow for a better understanding of dynamic microbial processes, such as food fermentations and food spoilage, allowing the food industries to become more competitive by optimizing microbial activities and also by reducing wastes. Furthermore, the possibility to understand foodborne pathogens behaviour in the food chain, will make their control more precise identify strategies which will decrease the burden of foodborne diseases. Future work will surely benefit of such interesting chances to gain fundamental insights from the food microbiome studies that will further change the overall approach to food science, in the same way as the human microbiome is doing with clinical research.

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Figure legends

Figure 1 – Evolution of the microbiological approaches used to study microbial diversity and ecology in food ecosystems.

Figure 2 - Sourdough-microbe networks based on RNA data obtained by pyrosequencing of 16S rRNA gene amplicons. The microbial evolution of three sourdoughs, based on different cereal flours (rye [diamonds], durum wheat [circles], and soft wheat [triangles]), is shown from the first step of spontaneous fermentation (red symbols) until stable sourdoughs were obtained. The microbial diversity decreases and the number of shared operational taxonomic units increases as the propagation proceeds (green, blue, fuchsia, and orange symbols correspond to sourdoughs after 1, 2, 5, and 10 days of propagation, respectively), making the three final sourdoughs very similar (Copyright © American Society for Microbiology, Applied and Environmental Microbiology, volume 79, 2013, pages 7827-7836, and DOI: 10.1128/AEM.02955-13).

Table 1. Relevant research contributions exploiting trascriptomics to investigate virulence, stress response and adaptation *in vitro* and *in situ* of foodborne pathogens.

Foodborne pathogen	Molecular method	Methodology	Aim of the study	Reference
Listeria monocytogenes	microarrays	*in vitro*	Adaptation to organic acids	[39]
Staphylococcus aureus	microarrays	*in situ*	Stress and adaptation in cheese	[40]
Escherichia coli O157:H7	microarrays	*in vitro* and *in situ*	Behavior in synthetic broth and food simulated system	[41]
Listeria monocytogenes	microarrays	*in vitro*	Response to acidic conditions	[42]
Bacillus cereus	microarrays	*in vitro*	Resistance to disinfectants	[43]
Listeria monocytogenes	microarrays	*in situ*	Expression of virulence, stress and adhesion genes in meat juices	[44]
Listeria monocytogenes	microarrays and RT-qPCR	*in vitro*	Response to salt stress	[45]
Listeria monocytogenes	RT-qPCR	*in situ*	Stress response during sausage fermentation	[46]
Listeria monocytogenes	RT-qPCR	*in situ*	Expression of virulence genes in salmon stored at differen temperatures	[47]
Staphylococcus aureus	RT-qPCR	*in situ*	Enterotoxin gene expression in cheese	[48]
Listeria monocytogenes	RT-qPCR	*in situ*	Salt stress response in liver pates	[49]

Figure 1

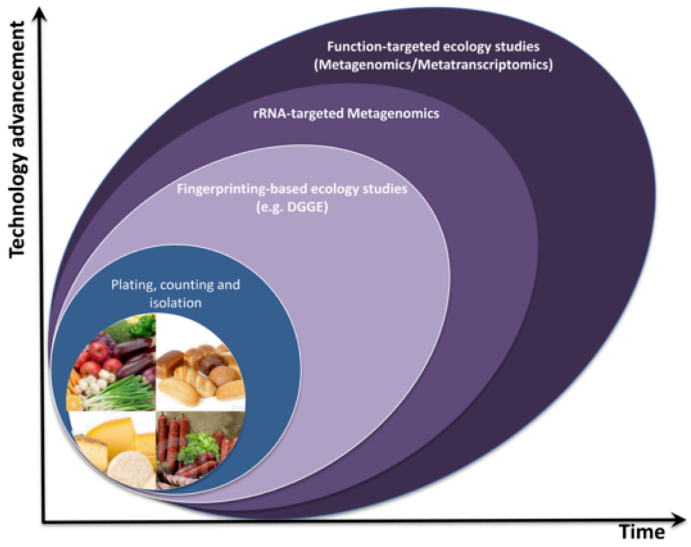
Technology advancement
Function-targeted ecology studies
(Metagenomics/Metatranscriptomics)
rRNA-targeted Metagenomics
Fingerprinting-based ecology studies
(e.g. DGGE)
Plating, counting and
isolation
Time

Figure 2