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A new perspective on microbial landscapes within food production

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Abstract

High-throughput, ‘next-generation’ sequencing tools offer many exciting new possibilities for food research. From investigating microbial dynamics within food fermentations to the ecosystem of the food-processing built environment, amplicon sequencing, metagenomics, and transcriptomics present novel applications for exploring microbial communities in, on, and around our foods. This review discusses the many uses of these tools for food-related and food facility-related research and highlights where they may yield nuanced insight into the microbial world of food production systems.

Introduction

The beneficial and detrimental contributions of microbes to food production, stability and flavor have been examined for centuries. Indeed, this scientific path led to many of the key discoveries in microbiological science from Pasteur's work demonstrating that yeast ferment grape juice to make wine [1], to Lister's demonstration that an isolated ‘*Bacterium lactis*’ strain was capable of souring (i.e. fermentation of) milk — an early illustration of a single microbial cause for infectious disease [2]. Thus the relationship of microbes to the foods we eat has had a long and notable scientific history, enabling the development of control measures to constrain and/or encourage specific microbial activity within food production to make safer, healthier and more flavorful products. A good example of this is the prevalent use of starter cultures in the production of various fermented products such as wine, beer, and cheese which both promote a controlled and efficient fermentation while simultaneously creating an environment that prevents growth of spoilage and/or pathogenic microbes.

Since the time of Pasteur and Lister, most of the scientific efforts in food microbiology have been necessarily reductionist in nature, identifying key microbial players that influence or infect foods and characterizing their biology and ecology. However, the tremendous advance in the study of microbial ecosystems in the last 20 years has spawned a new revolution in food microbiology. New tools and techniques to study microbial communities are increasing

the throughput and sensitivity with which we can approach food ecosystems. These newfound capacities are answering old questions while bringing new ones into focus, enabling exploration of microbial communities across time and space on a scale unimaginable a decade ago. They are improving the sensitivity with which we can probe already well-characterized systems as well as discover new ones. They are increasing sample throughput, enabling systems-level investigations and promoting meta-analyses. Furthermore, they also allow us to view food microbiology not as an isolated phenomenon but also as part of a complete ecosystem, answering questions about microbial source-tracking, co-evolution, and inter-connectedness with human health.

New tools

The advent of massively parallel, high-throughput sequencing technology (sometimes referred to as next-generation sequencing) is the revolution that sparked this sea change in our ability to conduct microbial ecology research. Several platforms and chemistries exist (e.g. Illumina, 454/pyrosequencing, ion semiconductor, and nanopore sequencing) but all employ nanotechnology to tether individual strands of DNA and detect the incorporation of individual nucleotides into each strand during polymerization events. Each system has its strengths and weaknesses, including different sequence read lengths, number of strands sequenced, and error rates [3] — but each has been a stepping stone in advancing our ability to investigate the inner workings of the microbial realm. This bodes well for the food sciences, bringing manifold improvements over earlier mixed- microbial detection techniques [4]

These new sequencing tools rely on the analysis of a single core molecule — DNA (and by transcription RNA) — yet possess many applications for microbial ecology analysis. The first is **amplicon sequencing** (reviewed in [5^{***}]), whereby marker-genes are amplified from mixed genomic DNA by PCR, sequenced directly, and aligned against a reference dataset to identify the taxonomic composition of whole microbial communities. This same process can also be applied to RNA (reverse-transcribed to cDNA) to profile the actively transcribing community within a sample. The taxonomic information provided by amplicon sequencing is frequently lower-resolution than that delivered by metagenome sequencing (which enables reconstruction of full-length marker genes) but is substantially higher throughput, facilitating exploration of massive numbers of unique microbial communities.

The second tool is **metagenome sequencing** (reviewed in [5^{***}]), or shotgun metagenome sequencing, whereby genomic DNA not from a pure isolate but from an entire, mixed microbial community is fragmented, prepared into a sequencing library, and sequenced. This can result in reconstruction of individual genomes or genome fragments for investigation and comparison of the genetic consortia and taxonomic composition of complete communities and their predicted functions.

Metatranscriptomics (reviewed in [5^{***}]), involves the same process as metagenome sequencing, but applied instead to RNA (reverse-transcribed to cDNA) from mixed-microbial samples. The result is a profile not of the mixed genomic content of a given

sample, but its mixed expression profile. This characterizes the expression behavior not of individual organisms but of entire communities in response to different conditions.

Application of microbial system-level tools in food production

Microbial community dynamics

Microbes play critical roles in the safety, stability, and nutrition of all foods at some level, whether they are a necessary processing component (as with fermented foods) or whether they deteriorate the shelf life and safety of fresh foods. Therefore, identifying, quantifying, and tracking the microbial consortia of food systems has long been a priority in food research.

Tracking complex microbial communities is fundamentally important in food fermentations, in which mixed microbial communities are inherently involved in the necessary transformations as well as spoilage of the product. Thus, high-throughput sequencing technologies are providing the greatest advance to this sector of research, where the increased sample throughput and detection sensitivity support analysis of larger and more complex studies. Amplicon sequencing approaches have been applied to study the microbial ecology of pearl millet fermentations [6], various Asian vegetable fermentations [7–9], seafood fermentations [10], Chinese liquor [11], cheese [12–14], sake [15] and other rice beers [16], olives [17], sourdough [18], kombucha [19], kefir and similar fermentations [20,21], wine [22,23], and spontaneously fermented American coolship ale fermentations [24,25]. What types of questions can be answered from describing the microbial succession in these fermented foods? Many of these fermentations involve undefined starter cultures (e.g. traditional cheese starters) [14], undefined, adventitious microbiota (e.g. coolship beers) [24], and/or multiple stages with mixed consortia (e.g. rice wine fermentations) [15,16]. Describing the normal and abnormal microbial states at different stages of these fermentations is important to characterizing consistency, identifying biomarkers for product quality or spoilage, diagnosing problem fermentations, and learning to manipulate fermentation conditions for improved process control. For example, we characterized the microbial consortia of American coolship (lambic-style) beers, which are fermented entirely by adventitious microbiota over the course of 3 years [24]. Comparing multiple batches allowed us to identify the ‘normal’ microbial succession in these beers and identify off-batches. Amplicon sequencing can also be used for biosurveillance of interesting microbes in fermentations and to design culture strategies for isolating these organisms that would not otherwise be detected by broad-spectrum culture methods [22].

The utility of these tools is by no means restricted to fermented foods and indeed they have been applied to study the microbial communities and stability of fresh and non-fermented foods. Amplicon sequencing has been used to study fresh fruits and vegetables [26], raw and pasteurized milks [27], poultry [28], beefsteaks [29*], and Chinese marinated pork hoof [30]. Investigating non-fermented foods with these tools offers non-targeted insight into microbial conditions post-harvest and post-processing, with implications for food stability and safety.

Metagenome sequencing offers several advantages over amplicon sequencing. First, as whole genomes are sequenced directly (following fragmentation and library preparation),

the possibilities for PCR amplification bias and other methodological biases are minimized. Amplicon sequencing relies on amplification of marker DNA sequences with ‘universal’ PCR primers; however, it is difficult to design primers that are equally homologous across broad phylogenetic groups, such as 16S rRNA primers for all Bacteria [31] or ITS primers for all Fungi [25]. As a result, some transcripts may be preferentially amplified while other clades (including uncultured clades) may be completely unrepresented [32]. Second, as metagenome sequencing does not require the use of PCR primers, it can be used to track groups for which universal PCR primers have not or cannot be designed, such as viruses. Third, full-length marker genes can be reconstructed from metagenomic data, allowing much more accurate taxonomic identification at species level compared to the segments currently covered by amplicon sequencing (which is limited by the read length of the sequencing platform). Finally, metagenomes can be used to track the frequency with which different gene classes are observed in samples, and hence the genetic capacity of the complete microbial community. However, meta-genome sequencing suffers from one major disadvantage relative to amplicon sequencing: as many entire genomes are being sequenced from each sample, the throughput is very low and the cost many-fold that of amplicon sequencing. This makes amplicon sequencing much more attractive and useful for large-scale studies, whereby thousands of samples can be analyzed simultaneously. A present, metagenomic sequencing is most useful for smaller, specific experimental questions involving a smaller number of samples.

In kimchi fermentations, metagenome sequencing revealed the involvement of a complex bacterial cohort and changing gene-family compositions at different stages of fermentation [33]. Several putative phage contigs were also detected in the metagenome, suggesting an increasing involvement of phage dynamics during the course of kimchi fermentations [33]. Indeed, metagenome sequencing reveals a rich tapestry of viral communities in different fermented foods, primarily dominated by bacteriophages [34]. Complex microbial communities including bacteriophages have also been detected in a single cocoa bean fermentation metagenome [35]. Such detection of viruses represents another useful output of metagenomics, as universally conserved marker-genes (e.g. 16S rRNA in bacteria) do not exist across viruses, inhibiting the use of amplicon sequencing for ‘universal’ viral detection. This application is ripe for exploration of the interplay between viral and cellular microbial communities in a range of food products, including other fermented foods where phage activity plays a role.

Metagenomic analysis can also provide keen insight into the functional attributes of a microbial populations associated with flavor production. Wolfe *et al.* [36•] extensively profiled the fungal and bacterial populations within the rinds of natural, bloomy and washed rind cheeses. Functional reconstruction of the metagenomes of cheese rinds demonstrated an enrichment of specific amino acid metabolism pathways in washed rind cheeses correlated with the well-known aromas associated with these cheeses. This analysis also revealed that the specific enzyme which converts methionine to methanethiol (a pungent volatile sulfur compound) resided within a bacterial population, *Pseudoalteromonas* sp., that was previously not overtly linked to flavor development in cheese.

In another novel use of metagenome sequencing, genome reconstructions have been used to tailor culture media for isolating uncultured microbes [37], albeit not in food fermentations. Nevertheless, this may have many applications toward foods, particularly as sequencing methods continue to detect organisms that have not been previously cultured from some foods.

Metatranscriptomics is another important, yet under-utilized, tool for food microbial ecologists. These analyses provide information not only on the taxonomic composition of samples, but also on active populations and the specific gene families that are expressed under different conditions, different times, and different locations. Such is the advantage of metatranscriptome analysis — in addition to the advantages offered by metagenome sequencing, metatranscriptome sequencing focuses solely on the transcriptionally active portion of the population, describing changes in behavior as well as community structure. At the time of this writing, only one study [38] has described sequence-based metatranscriptomics for a food (to our knowledge), in kimchi. Metatranscriptome analysis reveals that *Leuconostoc mesenteroides* is most active early in kimchi fermentations, with other *Leuconostoc* species, *Lactobacillus sakei*, and *Weissella koreensis* becoming dominant later in the fermentation. Expression profiles exhibit a decrease in protein metabolism over time, coupled with an increase in vitamin synthesis, stress tolerance, and carbohydrate metabolism, especially of lactic acid fermentation pathways. Importantly, transcriptome analysis revealed that the transcriptionally active community of kimchi fermentations had a slightly different structure from the composition revealed by amplicon sequencing [38]. A number of earlier microarray-based metatranscriptome studies in kimchi [39] and sourdough fermentations [40] have similarly demonstrated the insight afforded by this type of analysis. However, meta-transcriptome sequencing overcomes several disadvantages of microarrays, including probe target limitations for detecting undescribed and untargeted biodiversity in uncharacterized samples and false-positive results from cross-reactive microarray probes [41]. Metatranscriptome analysis in general poses many advantages for food microbial ecology studies, and many observations that cannot be captured by amplicon or metagenome sequencing. However, similar to metagenomics, the sample throughput of metatranscriptomics is much lower than amplicon sequencing, as the aggregate expression profiles of many organisms are sequenced in any given sample.

Food building ecosystems

The microbiota of man-made structures (referred to as the ‘built environment’) is of broad and growing interest lately due to its influence on various contributors to human health (air quality, toxin production, allergy promotion, pathogen spread) [42]. As the majority of the human diet in developed countries is processed, packaged, stored, and transported indoors for extended periods under essentially artificial conditions, food-processing environments and their associated microbial ecosystems are of tremendous importance to food quality and safety. The selective pressures exerted by building materials, substrates, and physiochemical conditions on the omnipresent microbial milieu contribute to the quality of the final product. This growing field of ‘built environment’ microbiology has been enabled by the advances in DNA sequencing ability, as well as the advent of ‘big data’ technologies such as the monitoring of environmental conditions and the analysis of large, diverse datasets. Methods

of incorporating microbial data and metadata have matured over the past decade, and hold immense promise to answer questions which were previously impossible to consider [5**]. Indeed, initiatives such as the Alfred P. Sloan Foundation's Microbiology of the Built Environment program have sought to take advantage of these new abilities to tap the potential of the field [43].

Aside from hospitals, few buildings have stricter building code requirements to minimize transmission of harmful microbes to humans than food facilities, including both food processing facilities and restaurants. Though the importance of controlling microbial sources, vectors, and reservoirs in food processing facilities have been understood for more than a century, high-throughput, non-targeted analysis can reveal the behavior of complete microbial communities across much larger spaces than afforded by the lower-throughput techniques of the past, yielding perspective on global population dynamics within food facilities as microbial ecosystems.

Detailed guidelines for building layouts, construction materials, and food processing practices (including sanitation) have evolved over the past century. The current incarnation of these policies in the United States is the FDA Food Code, which was first published in 1993 and is updated every four years (most recently in 2013), provides very extensive and detailed guidelines for facility construction, waste handling, product labeling, safe food storage, handling and preparation, and much more [44]. FDA and USDA guidelines cover both the design of the built environment, and processing procedures, to minimize transmission of harmful microbes from humans themselves, or from raw materials, into the final ready-to-eat products. These facilities and procedures are designed with an improved understanding of the sources, vectors, reservoirs, and characteristics of microbes in various types of food and beverage processing facilities. Other guidelines such as Sanitation Performance Standards pertain to ventilation, plumbing, sewage disposal, water supply, flow of operations, location of restrooms, dedicated hand wash sinks, building materials, and much more [45].

Our knowledge of how to control environmental conditions in the built environment to manipulate the indigenous microbiota, whether through original architectural design or periodic intervention, is still limited. However, air flow, temperature, cleaning procedures, and humidity are known to influence the microbial communities in man-made structures [46]. The origin of microbes, both undesirable, benign and beneficial, found in buildings is mostly unknown, though humans are known to be major microbial vectors to the spaces they inhabit [47].

Regardless of the original source of microbes, efforts to evaluate their impact will be facilitated by understanding their transfer through food processing facilities. Several investigators have used amplicon-based sequencing to investigate how food spoilage organisms move through the environments of cheese and meat processing plants [29,48]. In wineries, seasonal fluctuations alter the microbial communities detected on equipment surfaces [49]. The organisms on different equipment surfaces reflect the substrate encountered at that site, demonstrating bi-directional transfer of microbes between fermentations, vectored by key equipment surfaces. Likewise, in beefsteak processing plants,

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bi-directional transfer of bacteria occurs between carcasses, processed meat, and environmental surfaces, increasing the likelihood for product contamination [50]. In cheesemaking plants, substrates and processing steps shape the microbial communities of equipment surfaces, interacting with the developing fermentations [51]. The aging-room surfaces of different cheesemaking plants harbor distinct, indigenous microbial communities that dominate the surfaces of the cheeses aged therein, forming the basis for a ‘house’ microbiome involved in regional cheesemaking [51]. We see a similar situation in a *kimoto* (uninoculated) sake brewery, where the bacteria and yeast conducting these fermentations are vectored on equipment surfaces [15]. In beer breweries, season, substrate, and human processes all shape the microbial communities of equipment surfaces [52**]. This study of breweries also tracked the flow of hops-resistance genes through the facility, along with the information on the microbial community [52**].

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Outside of commercial facilities, domestic food preparation areas are also of interest to those who study the built environment microbiota. One study mapped the microbiota of kitchen surfaces [53], while others have investigated the impact of food preparation hygiene and cleaning [54,55]. In addition to the microbiota of food-preparation surfaces, the ambient bacterial levels of food storage areas such as refrigerators are also of note [56]. Several studies have looked at meat storage over time and under various conditions via pyrosequencing-based measures of the associated microbial communities [57–59]. Similar efforts have been made with vegetables [60–62].

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At present, no metagenomic or metatranscriptomic analyses in built environment food systems have been published. The lower sample throughput of these techniques currently limits their use for visualizing whole microbial landscapes. However, amplicon sequencing could be used for landscaping and sample dereplication, followed by targeted metagenome and metatranscriptome sequencing to observe changes at critical sites. Continuous improvements in sequencing technologies are bound to increase sequence yield and decrease cost, increasing the sampling capacity of these techniques.

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In lieu of food-associated studies of these types, non-food associated built environment studies are illustrative of what is currently possible. Strain-level monitoring of pathogens or spoilage organisms in a food-associated environment is now possible, as has been done in sewage systems and neonatal intensive care units via metagenomics techniques [63,64]. Correlating behavior of food preparation employees with the aggregate microbial transfer through the food processing environment may reveal contamination sources [65*]. A recent study examining the shifting microbiome of a metropolitan subway [66] illustrates how large-scale studies of food supply chains could be possible. Input ingredients to food facilities that contain spoilage or pathogenic microbes may be identified using the same metagenomic methods used to identify marine microbes uniquely found in flooded subway stations. Evaluating the influence of contaminated ingredients is analogous to tracking the flow of genes and strains through the built environment, as has been done with methicillin resistant *Staphylococcus aureus* (MRSA) [66].

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New bioinformatics tools are also improving the opportunity to examine food facilities. Software such as SourceTracker enables the detection of directionality of transfer of

microbes or genes [67]. By profiling various brewery substrates (grain, hops, yeast and beer) as well as public human-associated microbiomes, Bokulich *et al.* [52•] employed SourceTracker to visualize global contamination routes within a brewery (Figure 1). This type of tracking has also been used for investigating the source of hospital acquired infections [68•], and to complement older methods of tracking target genes through an environment, such as monitoring carbapenem resistance in hospitals through whole genome isolate sequencing [69].

A good example of incorporating metadata into a built environment study can be found in a recent study of a hospital microbiome [70]. With the use of extensive metadata such as relative humidity, humidity ratio, indoor dry-bulb temperature, illuminance, differential pressure between rooms and hallways, human occupancy and activity in the patient rooms via indoor air CO₂ concentrations and infrared doorway beam-break counters, outdoor air fractions in the heating, ventilating, and air-conditioning systems, this study is a model for fine-grained analysis of the effect of environmental conditions on the microbiota. Many of these measurements were made at 5-min intervals for almost one year, providing a total of 8×10^6 data points. This massive number of observations demonstrates the detail at which researchers are currently able to monitor our built environments and would be of clear relevance to food production facilities.

A new concept of ‘microbial terroir’

Microbes play important roles in food quality and safety both pre-harvest and post-harvest. Consequently, understanding what factors shape the microbial consortia of agricultural products may yield benefits to any food. In California wine grapes, we observe clear microbial patterns associated with regional, varietal, and climatic factors [71•]. Soil serves as a reservoir for many of the bacteria that colonize grapevine surfaces [72], and vineyard soils exhibit similar microbial biogeographic patterns, suggesting that edaphic factors also influence this phenomenon [73]. Many of these regionally discriminant bacteria and fungi have well established roles in grape and wine qualities, including potential grapevine pathogens, wine spoilers, and beneficial fermentative organisms [71•]. Hence, these patterns potentially contribute to the expression of regionally unique wine characteristics, or *terroir* [74]. This concept was recently extended by Knight *et al.* [75•] who performed a chemical analysis on Sauvignon Blanc fermentations carried out by specific *Saccharomyces cerevisiae* subpopulations previously shown to be regionally distributed within six of New Zealand's wine growing regions. These different *S. cerevisiae* strains drove different metabolic outputs in the resultant wines, showing, for the first time, a direct link between regional microbiota content and the chemical attributes of a finished wine.

Does a similar microbial terroir exist among other fermented products? Some dairy fermentation products do exhibit a similar regional character. Bokulich *et al.* [21] demonstrated that both production region and milk type influence the microbiota content of fermented matsoni (a fermented milk in the Caucasus region), suggesting that the traditional production methods preserve the transfer of unique regional microbiota from batch to batch. However, other aspects of dairy products do not show similar regionality. Wolfe *et al.* demonstrated that cheese rinds from geographically distant regions (Europe and United

States) have quite similar microbial communities whereby environmental factors (i.e. humidity, pH, salinity) appear to be more important drivers of rind community composition [36**]. Further elaboration of the relationship between regional traits (e.g. climate, agricultural traditions), plant and/or animal biology, microbial patterns, and food product qualities may enable greater control and decision-making in agriculture with consumer-oriented goals in mind. Dissolving the disconnect between agricultural decision-making and product qualities holds promise for improving the supply, security, consumer acceptance, and economic value of important agricultural commodities in the face of a burgeoning global population.

Future directions

As the cost of sequencing continues to plummet and the speed at which data can be interpreted skyrockets, the point at which the value proposition of microbial monitoring of food and food preparation systems justifies its expense is rapidly approaching. We anticipate these analyses will reveal new layers of the microbial dynamics of food ecosystems. Investigating the effects of environmental conditions, cleaning strategies, processing conditions, human activities, and substrates on the composition and especially the expression profiles of ambient microbial communities offers exciting possibilities for understanding microbial behaviors in food processing. Moreover, comparison of many different food facilities microbiomes on a global scale using tools such as Qiita (qiita.microbio.me) may allow more generalized conclusions.

The mounting evidence suggests that a complex scenario plays out in food processing facilities: production activities and environmental conditions in all steps of the production chain shape the microbial communities inhabiting surfaces and interacting with foods in these spaces. As many of these processes are human-controlled, it may be possible to actively cultivate beneficial microbial communities within the processing environment, though precise controls for microbial community structuring have yet to be determined. The food facility of the future may be envisioned as a ‘smart’ ecosystem, which incorporates thoughtful architectural design cognizant of the flow of microbes through the system, continual systems level monitoring of potential contamination points, evidence-backed targeted interventions where issues are identified, and rapid quality assurance of the final product. The insights obtainable from such data-rich facilities have the potential to innovate equipment and facility design, sanitation practices and routine microbial monitoring, leading to safer, more efficient, and sustainable food-production practices.

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Figure 1.

Mapping microbial contamination sources inside the brewery as presented in Bokulich *et al.* [52•]. Maps of brewery indicate the predicted relative contamination of brewery surfaces by incoming microbial sources (i.e. grains, hops), estimated by SourceTracker [67]. Increasing color intensity of each surface indicates an increasing relative degree of microbial contamination from that source type.