# Next-Generation Food Research: Use of Meta-Omic Approaches for Characterizing Microbial Communities Along the Food Chain

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**Abstract**

Microorganisms exist along the food chain and impact the quality and safety of foods in both positive and negative ways. Identifying and understanding the behaviour of these microbial communities enables the implementation of preventative or corrective measures in public health and food industry settings. Current culture-dependent microbial analyses are time-consuming and only target specific subsets of microbes. However, the greater use of culture-independent meta-omic approaches has the potential to facilitate a thorough characterisation of the microbial communities along the food chain. Indeed, these methods have shown potential in contributing to outbreak investigation, ensuring food authenticity, assessing the spread of antimicrobial resistance, tracking microbial dynamics during fermentation and processing, and uncovering the factors along the food chain that impact food quality and safety. This review examines the community-based approaches, and particularly the application of sequencing-based meta-omics strategies, for characterizing microbial communities along the food chain.

## INTRODUCTION

Microorganisms along the food chain from farm to fork influence food quality and safety. Historically, culture-based techniques have been used extensively to characterise these microbes. However, with the development of molecular methods and high-throughput sequencing technologies, culture-independent techniques have become more relevant to food microbiome analysis. This has resulted in a corresponding shift from the investigation of specific taxa or groups of food microorganisms to a broader community-based analysis (Cocolin and Ercolini 2015). These methods are based on the extraction of nucleic acids, proteins, and/or metabolites, allowing for the detection and characterisation of microbes within an environment without the need for culturing. As the occurrence of and interactions between microorganisms impact the quality and safety of food, a deeper understanding of these processes allows for early interventions to adverse food safety events, ensuring optimal food quality, and identifying the source of desirable or undesirable microorganisms.

Despite being labor-intensive and time-consuming, culture methods are still the methods employed most regularly in the food industry (Dwivedi and Jaykus 2011, Sohier et al. 2014). However, one of the biggest limitations is that these approaches frequently detect only a fraction of the microbes that are present in the sample as they rely on the isolation and growth of single microbes on culture media whose metabolic and physiological requirements can be reproduced *in vitro*. This not only overlooks the portion of microbes that are viable but not culturable (VBNC) but also fails to consider the relationships within the community of bacteria present in the sample (Cao et al. 2017). Uncultured microorganisms are estimated to account for up to 99% of the microorganisms in many environments, meaning that the use of traditional culture methods causes a gross underestimation of the microbial population (Handelsman 2004). Although this level of underestimation may not be as considerable in food systems, nevertheless, because the microbiomes of food and food processing environments are composed of complex, dynamic microbial communities, meta-omic approaches have the potential to provide a more accurate and greater understanding of these communities.

In this review, the contribution of microorganisms to the quality and safety of food and the traditional approaches to microbial characterisation are briefly described. The main focus of the review is on sequencing-based meta-omic approaches and their contribution to understanding microbial community dynamics in food, food-associated environments and along the food processing microbiome. Other non-sequencing-based meta-omic approaches are also mentioned in brief.

## IMPORTANCE OF MICROORGANISMS THROUGH THE FOOD CHAIN

### Quality: Flavor, Texture, Fermentation and Spoilage

Food quality is often associated with physical parameters such as pH and moisture content, which can influence the growth and survival of microorganisms within a food and the food chain. Food spoilage is a process or change that renders a product undesirable or unacceptable for consumption, which is impacted by both the food’s intrinsic characteristics and the extrinsic environment (Blackburn 2006). It is a complex process, whereby food undergoes biochemical changes, often due to microbial activity according to ecological determinants (Nychas and Panagou 2011). Some common spoilage bacteria include *Pseudomonas* spp., *Shewanella* spp., *Bacillus* spp., *Clostridium* spp., lactic acid bacteria (LAB), and Enterobacteriaceae (Blackburn 2006). Ultimately, different bacteria cause varying quality problems in different types of food, with some examples presented in Table 1.

Furthermore, despite their slower growth rate, yeasts and molds are able to exploit many ecological niches in food systems and can utilise substrates and tolerate extreme conditions that are not possible for bacteria (in't Veld 1996, Petruzzi et al. 2017). Common spoilage yeasts include species of *Zygosacchromyces* (in high sugar foods), *Saccharomyces* (a cause of gassiness and turbidity in wines), or *Candida* (cause off-flavors in meat and dairy products) and common spoilage molds include *Zygomycetes* (in produce with high water content), *Penicillium* spp. (cause rot in fruits), and *Aspergillus* (in grains, spices and nuts) (Sahu and Bala 2017).

However, microbes can also improve food quality by changing its intrinsic characteristics. This is evident in fermented foods, where the activity of microbes can improve their organoleptic and nutritive qualities in addition to extending shelf life. Fermented food microbes can either be introduced spontaneously (from the raw materials or production or processing environments) or inoculated as starter cultures and, over time, can produce enzymes, volatile compounds, and antimicrobial molecules, such as organic acids, fatty acids, hydrogen peroxide, diacetyl, and bacteriocins, which can help to slow down or prevent the growth of spoilage and pathogenic microbes (Reis et al. 2012). Although the spontaneous introduction of microbes is of specific relevance to this review, here we briefly provide an overview of some of the most important microorganisms in fermented foods in general.

LAB are among the most important microbes in the production of several fermented foods (Hatti-Kaul et al. 2018). This is reflected in the natural adaptation of many LAB to fermented food environments but has been complemented by many years of research to better understand and enhance their contribution to product safety as well as organoleptic, nutritional, and health properties (Leroy and De Vuyst 2004). Different species of LAB have been used in dairy products (cheese and fermented milks), meats (sausage), fish, vegetables (sauerkraut and pickles), soy sauce, cereals (sourdough), and alcoholic beverages (wine) (Leroy and De Vuyst 2004). Another group of bacteria associated with fermentation are acetic acid bacteria, which mainly consist of *Acetobacter* and *Gluconoacetobacter*. This group of bacteria play important roles in coffee, cocoa and vinegar fermentation because of their ability to oxidize carbon substrates (Schwan and Ramos 2014). *Bacillus subtilis* and *Bacillus licheniformis* are important for the industrial-scale fermentation of soybeans as they grow rapidly, resulting in short fermentation times (Schallmey et al. 2004). Yeast can also play an important role in the production of many fermented foods. *Saccharomyces cerevisiae* is used in alcoholic fermentation, and yeasts are ultimately used in many indigenous fermented foods as they are acid tolerant, able to grow at high temperatures, and are present in many environments (Schwan and Ramos 2014). In Asia, indigenous foods fermented with yeast, such as miso, soy sauce and wines, are commonly consumed (Aidoo et al. 2006).

**Safety: Pathogens and Microbial Antagonism**

Despite the value of fermented food and other microbes in contributing to food quality and safety, with respect to food safety, microbes are frequently regarded negatively, with foodborne pathogens responsible for foodborne illness and outbreaks across the globe annually. The consumption of contaminated food causes an estimated 4,500 deaths annually in Europe (World Health Organisation 2017). The causative agents of foodborne outbreaks in Europe in 2019 were bacterial pathogens (26.4%), bacterial toxins (19.3%), viruses (10.7%), and parasites and other agents (3.6%), and 40% of reported outbreaks had unknown causative agents (European Food Safety Authority and European Centre for Disease Prevention and Control 2019). Common pathogenic bacteria include *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringens*, *Cronobacter sakazakii*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *Vibrio* spp., and *Yersinia enterocolitica*. Viruses such as norovirus and hepatitis E as well as parasites, including *Toxoplasma gondii* and *Trichinella spiralis*, are also common causes of outbreaks and have been recently reviewed (Bintsis 2017). There are various ways that pathogenic microorganisms can enter the food chain. They can be inherent to the raw ingredients, or introduced along the processing line via equipment, food handlers or packaging materials, among other routes. Microbial communities can also be present in the form of biofilms, which are microbial communities that adhere to solid surfaces and may contain pathogenic and spoilage species that can persist on surfaces in food-processing facilities (Coughlan et al. 2016). Once attached, these biofilms can be difficult to remove as they are embedded in a polymeric matrix and cells in the biofilm may be resistant to disinfectants or antimicrobials, particularly in mixed-species biofilms (Yuan et al. 2020). Research efforts on control strategies to prevent biofilm formation and remove existing biofilms are ongoing to overcome this challenge in the food industry. Food safety management systems, including hazard analysis and critical control points, and risk assessment principles have been widely implemented to prevent foodborne illnesses and outbreaks and control the spread of pathogens along the food chain. However, these management systems are reliant on having a thorough understanding of the microorganisms present and the risk they may pose.

Although microbes are often viewed negatively from a food safety perspective, some have been useful in biocontrol or biopreservation. Microbial antagonism has been applied in the food industry through the use of bacteriocins, phages, and more (Jordan et al. 2014). Bacteriocins, which consist of antibacterial peptides, have been used to target spoilage and pathogenic bacteria in food and in turn prolong the shelf life and improve the safety of food (Galvez et al. 2008). Bacteriocins from LAB such as nisin has been approved for use in foods and is most commonly used in foods such as meat, dairy and vegetable products (Jordan et al. 2014). Bacteriophages or phages are virus predators of bacteria that have shown great promise as they are naturally occurring and control for specific pathogenic bacteria without impacting the quality and microbiota of foods (O'Sullivan et al. 2019). Phages have been applied to a range of foods at various stages from farm to fork to eliminate common pathogenic bacteria such as *Campylobacter jejuni*, *Salmonella* spp., *E. coli* O157:H7, and more (Vikram et al. 2020). Additionally, biofilms from some species can aid in improving food safety by outcompeting undesirable bacteria. Some LAB strains were found to exhibit antagonistic properties against unwanted bacteria, act as a natural barrier, and alter biofilm formation of spoilage microbes (Ouali et al. 2014). In food, the microbial community and the interactions between microbes play essential roles in food quality and safety.

## TRADITIONAL APPROACHES TO CHARACTERISATION OF MICROBES

As noted above, culture-based assays have historically been used for the detection, enumeration and isolation of viable foodborne pathogens or spoilage microbes in food and environmental samples (Dwivedi and Jaykus 2011). In general, samples are first homogenized and then often undergo enrichment steps (pre-enrichment and selective enrichment), followed by selective or differential plating to distinguish from other microbes present and, finally, confirmation with biochemical, serological, or other methods. Pre-enrichment is used to recover injured cells and dilute inhibitory compounds in food samples, whereas selective enrichment increases the concentration of a target pathogen while suppressing the growth of other microflora (Dwivedi and Jaykus 2011). These conventional methods are inexpensive but time consuming and labour-intensive and can take from 2-3 days to a week for inoculation, isolation and confirmation depending on the targeted microorganism (Mandal et al. 2011). Furthermore, owning to the possible presence of VBNC bacteria, false negatives may occur, which could mean the unsuccessful detection of pathogens or spoilage bacteria in food.

With the need for more timely detection of bacteria for foodborne outbreak response, rapid methods that replace conventional plating steps with faster immunology or molecular-based approaches have been investigated in depth and adopted more widely in recent years (Wang and Salazar 2016). High levels of sensitivity and specificity are needed for food pathogen detection (Feng 2007). Immunology-based methods like enzyme-linked immunosorbent assay (ELISA) based on the specific binding of antigens with antibodies, have shown potential but their lack of sensitivity and relatively high limit of detection [$10^3 – 10^5$ colony forming units (CFUs)/mL] has meant that enrichment is generally first required before detection. Nevertheless, when ELISA is coupled with nanotechnology, its application for food analysis has achieved greater sensitivity, specificity and stability (Wu et al. 2019).

Compared to traditional culture methods, nucleic acid-based techniques such as polymerase chain reaction (PCR), quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) require shorter time and through multiplexing, can facilitate concurrent detection, real-time monitoring and quantification of multiple microbial targets (Liu et al. 2017, Tao et al. 2020). Development and optimization of each assay is important, as complex food matrices may hinder nucleic acid extraction and contain inhibitors that may interfere with reactions in the assay. Particularly when multiplexing, primer design is crucial, as primer sets require similar annealing temperatures for a successful assay (Wang and Salazar 2016). Unfortunately, these methods, like immunology-based assays, often still require enrichment or concentration steps because of their limit of detection ($10^3 – 10^4$ CFUs/ mL), and as pathogens are often in low concentrations in foods, direct detection is difficult (Ceuppens et al. 2014, Wang and Salazar 2016). Another rapid method used largely in clinical settings is matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS), which analyzes signals from ribosomal proteins after ionization and time of flight detction that are distinctive for each strain, allowing for rapid microbial identification (de Koster and Brul 2016). Although these rapid methods are generally preferable to culture-based approaches, thorough validation is required by industry and regulatory bodies before routine adoption.

Although culture-based and rapid methods are useful in identifying microbes in complex food- or food environment-related samples for public health and commercial purposes, they create an unbalanced emphasis on specific microorganisms and, despite multiplexing, still only capture a small percentage of the microbial community as a whole (Fleet 1999). As microorganisms exist in communities, it is important to study them as such because the growth, survival, and activity of one species or strain may impact or be associated with the presence of another. Furthermore, from a practical perspective, approaches that could theoretically allow the simultaneous identification of all pathogens and spoilage microbes in a sample from the food chain could have a disruptive positive influence.

## META-OMIC APPROACHES: COMMUNITY APPROACHES

Advances in technologies have provided the opportunity for faster and superior characterisation of food chain microbiomes, with a shift toward replacing or supplementing culture-dependent methods with culture-independent, molecular-based methods (Sohier et al. 2014). Whole-genome sequencing has successfully complemented culture-dependent methods by providing deeper discrimination of microbial strains than previous typing methods, with regulatory bodies in the United States and Europe now including it as a tool for pathogen typing and antimicrobial resistance (AMR) surveillance (Rantsiou et al. 2018). In *E. coli* O157:H7 outbreak investigations, genome sequencing stood out from other typing methods, providing insights that enabled improved epidemiological case and cluster identification, geographical origin tracking, and information of potential emerging strains (Jenkins et al. 2019).

In contrast, culture-independent methods including the use of DNA sequencing technologies, have enabled identification and characterisation of multiple microbes in foods or along the food chain at the same time while also bypassing the need to culture microbes (Cocolin and Ercolini 2015). Some current community-based approaches are shown in Figure 1.

### Sequencing-Based Meta-Omic Approaches: Metagenetics, Metagenomics and Metatranscriptomics

Metagenetics, also known as amplicon sequencing, metataxonomics, metabarcoding and sometimes, 16S metagenomics or 16S rRNA gene sequencing, is a targeted approach that involves the amplification of marker genes from mixed genomic DNA by PCR, followed by direct sequencing and alignment against a reference database to identify the taxonomic composition of whole microbial communities (Franzosa et al. 2015). The 16S ribosomal RNA (rRNA) gene is most frequently used in the identification of bacteria, as it is universally found in bacteria, and the gene contains nine hypervariable regions, some or all of which can be targeted through amplification and sequencing to identify the corresponding bacterial taxonomy. A similar approach can be applied to fungi, through targeting the 18S or 23S rRNA genes or the internal transcribed spacer (ITS) regions of the rRNA operon.

Shotgun metagenomics, commonly referred to as metagenomics, is the untargeted genomic analysis of a population of microorganisms by sequencing the entire DNA sample extracted from a mixed microbial community (Quince et al. 2017). This method involves fragmentation of the sample DNA, followed by preparation of a library that is sequenced, with the resulting data analysed to provide information on both taxonomic composition and functional potential of the entire microbial community. Due to the untargeted nature of metagenomics, information relating to all categories of microbes, including bacteria, viruses, archaea, and single-celled eukaryotes like fungi, can be derived from the sample (Quince et al. 2017), assuming the DNA extraction method is appropriate. The lack of an amplification step removes the bias that metagenetics may have and has greater sensitivity, enabling taxonomic classification up to the strain level. Another advantage of shotgun metagenomics is the potential for the recovery, if sufficient sequencing depth is applied, of metagenome-assembled genomes (MAGs), which can provide more genomic information, revealing functional and safety-related properties of specific taxa (Bowers et al. 2017), and allow for the investigation of strain-level diversity in food-related microbial species such as LAB (Pasolli et al. 2020).

Metatranscriptomics relates to the untargeted sequencing of total mRNA isolated from a sample, which allows for the identification of transcriptionally active microbes in the sample, and may provide further insights into the potential functional characteristics of the microbial community. This approach reveals the microbes that are viable and, indeed, most active within a community while also enabling a deeper understanding of how microbial communities in complex food microbiomes or food-related environments interact with each other. This approach can also be used to look at *in situ* gene expression in food, collecting information on the metabolic activities potentially related to food fermentation and/or spoilage that are currently expressed in a food ecosystem. An additional advantage of metatranscriptomics is the ability to detect RNA-based viruses, including foodborne pathogens such as norovirus (Lewis et al. 2020).

**Other Meta-Omic Approaches: Metaproteomics and Meta-metabolomics**

Other non-sequencing community-based methods, i.e., metaproteomics and meta-metabolomics, also have the potential to be used in food microbiome studies. Metaproteomics is the large-scale study of the entire protein complement produced by microbial communities within a sample at a given time point, which can aid in linking genomic and transcriptomic data to biological function, deepening the understanding of phenotypic changes as conditions change (Soggiu et al. 2016). Metaproteomics provides information on the microbial communities and their abundances and functions, the interactions within the community, the changes in community metabolism and physiology, and the substrate utilization, carbon sources and assimilation pathways of the microbes in the sample (Kleiner 2019). Mass spectrometry is used for metaproteomics and its application in the food microbiome has mainly been in the characterisation of fermented foods such as fermented soybean and cheese (Soggiu et al. 2016, Xie et al. 2019). Meta-metabolomics, however, involves the use of chemistry, biochemistry, and bioinformatics to detect and analyse small weight metabolites in samples and provide insights into microbial phenotypic characteristics. There are two categories of meta-metabolomic analyses: untargeted, which focuses on the detection of as many groups of metabolites as possible, and targeted, which focuses on a specific user-selected group of metabolites under determined conditions (Li et al. 2020). Researchers in this field typically use either mass spectrometry or nuclear magnetic resonance to evaluate food ingredients, quality, safety, authenticity, and traceability (Kim et al. 2016). The integration of metaproteomics and meta-metabolomics with other omic approaches has been used to provide novel insights and link genomic information with phenotypes (Kim et al. 2016, Pinu et al. 2019).

**Current Challenges in Sequencing-Based Meta-Omic Approaches**

Despite their promise, these sequencing-based approaches have challenges to overcome to achieve wider application. The major challenge for these meta-omic approaches is the lack of standardization, causing variation in results because of the use of different extraction methods, sequencing platforms, databases, and bioinformatics tools. To highlight this point, we refer to several studies that have found differing conclusions depending on the approaches taken for analysis, thereby highlighting the need to identify those that provide the greatest accuracy and, ultimately, their use in a standardized manner (Lewis et al. 2020, McHugh et al. 2021, Walsh et al. 2018, Yang et al. 2020).

Similarly in terms of analysis, the fact that results are typically presented in terms of relative abundances may lead to misinterpretations, as an increase in the relative abundance of one taxon results in the concurrent decrease of others. For this reason, it is necessary to quantify microbial communities by using complementary methods and efforts to do so have included digital PCR, qPCR, flow cytometry and culture. It is notable that the use of synthetic standards in sequencing has produced varying results for different methods, again highlighting a need for caution during analysis (Galazzo et al. 2020).

Furthermore, for metagenetics, there is a difficulty when endeavoring to compare outcomes across different studies arising because of a lack of consistency relating to the hypervariable region of the 16S rRNA gene targeted (Claesson et al. 2010). Additionally, the focus on one marker gene can cause other issues. In particular, the operon copy number for 16S rRNA genes differs across taxa, which may inadvertently affect quantitative estimation. Single-copy target genes like *recA*, *rpoB*, and *gyrB* have been suggested as alternatives, but their use is limited because of their relevance to specific taxa of bacteria only and/or the absence of databases that are sufficiently populated (Ogier et al. 2019, Poirier et al. 2018)

From the perspective of methodology, extracting DNA/RNA of sufficient concentration and quality is essential for sequencing, which may be challenging in some circumstances, such as from environmental swab samples taken from areas with a low microbial load (De Filippis et al. 2020). Amplification methods such as multiple displacement amplification (MDA) have been applied to generate DNA of sufficient quantities and the inclusion of controls have been investigated to reduce contamination, but these methods can lead to biases (Marine et al. 2014, McHugh et al. 2021).

For both standard metagenetic and metagenomic sequencing, there is no differentiation between DNA extracted from living and dead organisms within a microbiome, which is of key importance with respect to food or food environment microbiome studies. Propidium monoazide (PMA) and the previously more commonly used ethidium monoazide (EMA) are

DNA-binding dyes that selectively bind to accessible DNA present in the matrix, essentially binding to DNA from dead bacteria and other cells and preventing its amplification during library preparation (Nocker et al. 2006). Treatment with PMA before DNA extraction thereby selects for the subsequent sequencing of DNA from viable cells. The successful application of PMA with sequencing allowed the selective analysis of viable cells during milk processing and cheese manufacturing (Erkus et al. 2016, Kable et al. 2019). However, its performance can be influenced by the microbial community and sample biomass (Wang et al. 2021). Research on the application of these dyes with the use of internal standards could provide insights that may allow for quantification of live and dead cells, and optimization of this treatment in different food or environment matrices. Another option is the sequencing of RNA in place of or alongside DNA, as measuring RNA copies targets the active microbial fraction, which allows the differentiation of viable and non-viable microbes (Mira Miralles et al. 2019), although the instability of mRNA can again provide challenges. Further research on the discrimination of live and dead cells is required, particularly for the application of these sequencing-based approaches for food-related samples.

It is also important to note that for metagenetics in particular, the short reads generated by some sequencing platforms, such as those developed by the market leader, Illumina, can be limiting with respect to assigning taxonomy at the species level. Other sequencing platforms that produce longer reads, such as those from Oxford Nanopore Technologies (ONT) or Pacific Biosciences, may address this but lower read accuracy and higher sequencing costs can be issues.

Although shotgun metagenomic sequencing overcomes many of the biases associated within amplicon-based approaches, one of its biggest challenge is the reduced microbial sequencing depth that occurs when randomly sequencing samples that contain high amounts of host DNA. Although most studies remove the reads from host DNA during bioinformatic analysis, a more efficient alternative is to deplete host DNA or enrich microbial DNA through various chemical methods and commercially available kits (Marotz et al. 2018, Yap et al. 2020). Similarly in metatranscriptomics, highly abundant rRNA can result in increasing costs and complex downstream analysis. To overcome this challenge, rRNA depletion or mRNA enrichment strategies before sequencing and/or post-sequencing removal during downstream analysis have been adopted (Shakya et al. 2019).

Additionally, with the use of sequencing technologies, advanced computational power and bioinformatics skills are necessary for their use, which add to the challenges when considering the application of these approaches.

## APPLICATIONS OF SEQUENCING-BASED META-OMIC APPROACHES ALONG THE FOOD CHAIN

### Public Health Applications

The sequencing-based meta-omic approaches mentioned above have contributed significantly to the study of various diverse microbiomes, including by facilitating significant advances in food microbiome research. From a public health perspective, these meta-omic approaches have provided insights relating to pathogen detection, outbreak investigation, AMR determination and food authenticity and source tracking.

**Pathogen detection and outbreak investigation.**

Meta-omic approaches are advantageous, as they bypass the need for culturing and enrichment of pathogens from samples before identification and characterisation of putative etiological agents. They also are able to reveal the presence of uncultured and hard to culture microbes, which may be useful in surveillance, source attribution, risk assessment and epidemiological analysis when traditional methods fall short (EFSA Panel on Biological Hazards et al. 2019). Both metagenetic and metagenomic approaches have enabled the detection and characterisation of pathogens in various foods, including vegetables, meat, and dairy products (Aw et al. 2016, McHugh et al. 2018, Mira Miralles et al. 2019, Yang et al. 2016). Metatranscriptomics, although less widely applied because of the challenges in RNA isolation, also has great potential for identifying viable pathogens in food (Yang et al. 2020).

Use of metagenomic approaches can extend beyond the food chain, where metagenomic sequencing of patient stool samples collected during the outbreak in Germany of STEC (Shiga toxin-producing *E.* coli) O104:H4 assisted the recovery of genomes of the outbreak strain (Loman et al. 2013). Moreover, metagenomics is useful when a viral agent is the cause of the outbreak or, in the case of multi strain outbreaks, it is able to discriminate and characterise several strains, allowing them to be distinguished considerably faster than traditional culture-based methods (Buytaers et al. 2020). Compared to metagenetics, which may be more useful for low biomass samples because of the amplification of the target, metagenomics facilitates more sensitive characterisation to the species level and further investigation of the functional potential of microbes present (Grützke et al. 2019).

Despite the potential of these meta-omic approaches, they are currently not widely used. One reason is the lack of harmonized methods and standardized, accredited workflows/pipelines that would allow consistent detection and characterisation of outbreak-causing agents (EFSA Panel on Biological Hazards et al. 2019). However, the usefulness of metagenomic analyses can be enhanced when they are complemented with further quantitative molecular assays, highlighting their effectiveness in determining pathogen contamination or outbreak events. A big technical challenge that hinders greater adoption of meta-omic techniques as a routine screening tool for pathogens is that these techniques are not always sufficiently sensitive (Leonard et al. 2015, Lewis et al. 2020). With low numbers of pathogenic cells in samples, substantial sequencing depth is required, particularly for shotgun sequencing, as samples contain DNA from other microbes or contaminants such as animal, plant of human DNA (Yang et al. 2016). With sufficient sequencing depth, shotgun metagenomics can be a faster and more valuable tool that provides more information than current conventional workflows, which permit linking food/environment outbreak-related samples with clinical samples (Buytaers et al. 2020, Grützke et al. 2019, Li et al. 2020). Although the complexity of various food matrices can be a challenge, this is not as great an issue for less biologically complex matrices, such as water used in food production or some minimally processed foods (Fernandez-Cassi et al. 2017).

**Identification of antimicrobial resistance-encoding genes.**

Over the past decades, AMR has been identified as a serious public health threat and because of this, more tools have been published for the detection of genetic determinants of AMR from sequencing data. Although whole-genome sequencing of cultured isolates is usually utilized, metagenomic sequencing shows great potential for monitoring AMR, as it has out-performed culture-based methods in quantifying resistance in swine herds (Munk et al. 2017). Shotgun sequencing has shown success in the monitoring of AMR genes in the environment from farm to slaughter (Noyes et al. 2016, Pitta et al. 2016). It has also been used to understand the association between antimicrobial use and resistance and the effect of processing on the resistome and virulome (Campos Calero et al. 2018, Mencía-Ares et al. 2020, Van Gompel et al. 2019).

As with other metagenomic approaches, sequencing depth and the presence of host DNA should be considered, as they have been found to affect resistome profiling in environmental and food samples (Gweon et al. 2019, Rubiola et al. 2020). Other challenges include the difficulty in assigning ARG to their host species or strains, which may be addressed by sequencing with long-read technology and the choice of reference resistance gene database, where differences were found between gene variants from the same reference sequence from different databases, reiterating the need for comprehensive databases and standardized workflows (Doyle et al. 2020, Slizovskiy et al. 2020). It is also important to note that the AMR data may not always be phenotypically relevant, as these genes might not be expressed or the choice of bioinformatic tools can result in false positives or negatives (Doyle et al. 2020). From the perspective of gene expression, metatranscriptomics can potentially be employed to complement the analysis (Wang et al. 2020). The analysis of the mobilome (all mobile genetic elements of the microbiome) has also been paired with resistome analysis to understand the potential spread of AMR genes and virulence factors through horizontal gene transfer (Slizovskiy et al. 2020).

**Food authenticity.**

Food fraud is a global issue that has many consequences, including possible health risks, economic losses, and hindering sustainability efforts. Metabarcoding has been used to determine the authenticity and origin of honey, traditional Chinese medicines, fish, and more (Carvalho et al. 2017, Coghlan et al. 2012, Khansaritoreh et al. 2020, Liu et al. 2020). The basic concept is that the microbiome associated with a traditional food is closely linked to the geographical origin and mode of production of the food as the microbes are typical of raw materials and environment. Although there have been some successes, there are challenges associated with using microbiomes as a means of determining the provenance of food. These include the need for the existence of databases containing the components of the expected microbiome of the food and the potential alteration of the microbiome due to storage or processing conditions (Liu et al. 2020). Similar to other meta-omic applications, the reliance on the completeness of reference databases together with the accuracy of food matrix authentication are important to avoid inaccurate conclusions. Haiminen et al. (2019) found both DNA and RNA shotgun sequencing to be accurate untargeted methods for food authentication and contaminant detection, which has been applied by Kamilari et al. (2019) to characterise Protected Designation of Origin (PDO) cheeses with complementary metabolomics to define product origin differentiating factors.

### Other public health-related fields.

Besides the food industry, other fields have also found benefits in the application of community-based microbiome analysis methods. Community-based approaches have contributed to the increasing knowledge of the indigenous microbial community and AMR patterns in both healthcare settings and water systems that have provided evidence for the greater need for surveillance (King et al. 2016, O'Hara et al. 2017, Zhang et al. 2017). In hospital settings, meta-omic approaches have provided clues to the routes of entry and relationships between pathogens and non-pathogens, as well as helped in environmental surveillance to fight hospital-acquired infections and AMR (Comar et al. 2019, Rampelotto et al. 2019). Similarly, when supplemented with other techniques, shotgun metagenomics was effective in uncovering the presence of virulence factors and novel biomarkers of pathogen-related species in drinking water distribution systems (Zhang et al. 2017). Additionally, on an international scale, urban sewage and waste from aircraft flights have been cited as economically and ethically acceptable approaches for continuous global surveillance and prediction of AMR using metagenomics (Hendriksen et al. 2019, Petersen et al. 2015).

## Food Industry Applications

Microbial communities exist throughout the food chain and understanding their dynamics and the conditions that promote or hinder their growth would be useful for food safety and quality purposes. Research efforts using meta-omic approaches have looked into foods, food-associated environments, and food-processing steps, as presented in Figure 2, which are elaborated in the following sections.

### Foods: fermented and non-fermented.

One of the main applications of community-based approaches is in the study of fermented foods. Previous reviews noted that most of the early studies on fermented foods employed metagenetics to monitor the activity of microorganisms during fermentation (De Filippis et al. 2017). In recent years, more studies have utilized metagenomics and metatranscriptomics to understand the changes in microbial community diversity and activity during fermentation in a broad range of foods, including vegetables, cheeses, and more (De Filippis et al. 2016, Duru et al. 2018, Jung et al. 2013, Kim et al. 2020, Liu et al. 2020, Pham et al. 2019, Xiao et al. 2020). Metatranscriptomic analysis revealed the changes in gene expression and metabolic properties of LAB during fermentation of vegetables (Jung et al. 2013, Xiao et al. 2020). Likewise from metatranscriptomic analysis of cheese, metabolic interactions within the microbial community, and temperature-driven functional changes during ripening were revealed (De Filippis et al. 2016, Pham et al. 2019). The use of both metagenomic and metatranscriptomic analyses allowed for the detection of active microbes during fermentation and of microbes responsible for biogenic amine production in fermented soy products (Kim et al. 2020, Liu et al. 2020). This parallel approach was also useful in understanding the dynamics of the microbial community during ripening, revealing the impact of temperature on the microbial community and genes expressed (Dugat-Bony et al. 2015, Duru et al. 2018). These are selected examples of studies within the continuously growing pool of research that employ these methods to study the microbial consortia in fermented foods. Unsurprisingly, it has been suggested that multiple meta-omic approaches facilitate the improved, efficient, and sustainable production of fermented foods through detailed functional characterisation of their microbiomes (Chen et al. 2017).

Although the number of studies using meta-omic approaches to study non-fermented foods is considerably lower than that of fermented foods, those that have been completed highlight the great potential of such approaches. Most of these applications have related to the characterisation of food-associated environments or food-processing steps, which are elaborated in the following sections. Other than those studies, there have been promising studies involving the use of community-based methods to screen for spoilage or pathogenic microorganisms. However, because of the complex nature of food samples and the frequently low pathogen abundances, direct sequencing of DNA or RNA of food has, to date, been found to be less sensitive than conventional culture-based or amplicon-based methods (Lewis et al. 2020, Yang et al. 2020). It should also be noted that, even though both short and long-read sequencing technologies have shown promise with respect to accurate classification of microbes to the family and genus levels, not all approaches sufficiently classify to the species or strain level needed for pathogen detection (Grützke et al. 2019). This is sometimes a significant limitation, especially in terms of food safety, where identifying at only the genus level may not be informative enough to understand the actual species present that could cause food safety or quality issues along the food chain. The need for sensitive and specific tests coupled with other challenges prove that these community-based approaches are currently not applicable at the regulatory compliance level, but with further development that will be the standard be in the future (Yang et al. 2016).

**Food-associated environments.**

Food-associated environments, from farm to processing facility, have repeatedly been found to impact, both positively and negatively, the final product microbiome.

***Environmental factors.***

Microorganisms can enter the food chain at a number of different points. This includes the crops and animals from which the foods are sourced/derived as well as environmental factors such as soil, water, farming systems, pests, and climate conditions. Meta-omic approaches have found that factors such as pasture systems, animal housing, airborne dust, irrigation water, and several others can influence the microbiota diversity and composition of food (Allard et al. 2019, Doyle et al. 2017, Wu et al. 2019). Besides diversity and composition, meta-omic approaches used to characterise the resistome reveal that farm environments are potential vehicles for AMR bacteria and genes, originating from dust and animal feces that contribute to AMR spread and worker exposure (Luiken et al. 2020, Noyes et al. 2016). The use of animal waste as fertilizer (manure/wastewater) can also cause the dissemination of AMR bacteria and genes in the environment, which in turn affect the microbiota of crops grown or animals raised on the land (Allard et al. 2019, He et al. 2019). Seasonality is another contributing factor to the microbiota of the animal and plant environment. Seasonal impacts were evident in certain products, like milk and beef, where the use of metagenetics and metagenomics has revealed seasonal variations in the microbiota of final products (Hwang et al. 2020, Kable et al. 2016, McHugh et al. 2020).

***Food-processing environments.***

Meta-omic techniques have been adopted in the characterisation of several environments involved in the processing of foods such as meat (De Filippis et al. 2013, Hultman et al. 2015, Stellato et al. 2016), dairy (Anvarian et al. 2016, Doyle et al. 2017, Kable et al. 2016), and alcoholic beverages (Bokulich et al. 2015, Bokulich et al. 2013, Wang et al. 2018). One key observation from using meta-omic approaches for such studies is the presence of a resident microbiome that persists within the processing environments and has the potential to affect final food product quality and safety. This was highlighted in a recent review relating to the use of high-throughput sequencing to characterise the dominant taxa found in both processing environments and food products, which summarized the evidence that the processing environment can act as a reservoir and source of microbial transfer to food (De Filippis et al. 2020). This can be both beneficial and detrimental, with, for example, beneficial effects apparent in fermented food production. In this regard, microbes in the environment were found to contribute positively to the production of fermented vegetables, wine, and Chinese liquor (Bokulich et al. 2013, Einson et al. 2018, Wang et al. 2018). In contrast, spoilage or pathogenic microorganisms have been found on surfaces of various dairy-, meat- and vegetable-processing facilities using different meta-omic approaches (Hultman et al. 2015, McHugh et al. 2020, Pothakos et al. 2015, Stellato et al. 2016, Zwirzitz et al. 2020). For example, *Pseudomonas* spp. was found in drain biofilms in cheese- and salmon-processing plants (Dzieciol et al. 2016, Langsrud et al. 2016) and pathogens like *Staphylococcus* and *Yersinia* were found on surfaces in milk- and meat-processing plants (Hultman et al. 2015, Kable et al. 2019). Indeed, correlation of microbial communities in biofilms, as determined by metagenetics, with environmental factors has been used to track persistence over time, showing that bacterial communities were location-specific in meat- and fish-processing plants (Rodríguez-López et al. 2020). Additionally, microbial co-occurrences of pathogens with other microbes and microbial interactions within complex ecosystems can be evaluated through meta-omic approaches, which may determine patterns that favour or prevent the growth or survival of foodborne pathogens (den Besten et al. 2018, Illeghems et al. 2015). This was investigated through 16S rRNA sequencing that examined interactions between *Listeria* spp. and the microbiome within a food production facility, and identified species that acted as apparent protagonists or antagonists that had impacts on the presence of *L. monocytogenes* within the processing plant (Fox et al. 2014).

Handling can be a potential source of contamination or microbial transfer, whereby microbes can be unknowingly transferred from surfaces to the food product. *Moraxella* spp., a prominent meat-spoilage bacteria, was found on gloves of employees, which were identified as a potential source of contamination using full-length 16S rRNA gene sequencing throughout a pork-processing plant (Zwirzitz et al. 2020). Similarly, handling was identified as a catalyst in the proliferation of spoilage bacteria in beef products after high-throughput sequencing uncovered the origin of spoilage-associated bacteria from carcasses and their persistence in the environment (De Filippis et al. 2013).

**Food-processing steps.**

Using meta-omic approaches to monitor the changes in food microbiomes during food processing has been useful in understanding the impact of processes on the quality and safety of foods. This has been studied through two approaches. One approach has involved profiling the entire food-processing chain, where samples were taken from the start to the end of the process and meta-omic methods were used to track the changes in microbial community dynamics, which can facilitate the generation of mitigation measures. This whole-chain approach often involves sampling of both food and environmental samples and has highlighted areas where contamination or spoilage can potentially occur; e.g., in meat processing, animal carcasses or hides were identified as possible sources of contamination and measures taken during and after slaughter were found to be key in reducing bacterial load and transmission of AMR genes to meat products (Calero et al. 2020, De Filippis et al. 2013, Noyes et al. 2016, Yang et al. 2016). A similar approach to studying sausage production showed that the emulsification step selected for gram-positive spoilage bacteria (Hultman et al. 2015). Other investigations have highlighted the impact of storage, low temperatures, and equipment on the milk microbiota in dairy processing (Falardeau et al. 2019, Kable et al. 2016, McHugh et al. 2020), whereas in breweries, food contact surfaces were noted as areas that could allow transmission of spoilage bacteria or genes (Bokulich et al. 2015).

The other approach that has been taken when using meta-omic methodologies is process focused, where specific processing steps that are often considered critical points in food safety management systems are examined. Processes such as heat treatment, cold storage, packaging, cleaning, and others have been studied to understand the microbial dynamics during these processes and ensure their efficacy at eliminating or reducing growth of bacteria. Metagenetics used to investigate heat treatments unsurprisingly found a reduction of bacterial abundance and diversity in meatballs and cheese but it also affected the quality of the final products (Kamilari et al. 2020, Li et al. 2021). Similarly, monitoring the ripening processes of cheese using metagenomics and metatranscriptomics has provided a better understanding of the temperature-driven differences in flavor development (De Filippis et al. 2016, Duru et al. 2018), whereas metagenetics, proteomics and complementary physicochemical and sensory analysis revealed the efficacy of high-pressure processing in improving the quality and shelf life of fish fillets and led to the identification of quality markers for further study (Tsironi et al. 2019). For storage in particular, metagenetic and metagenomic analysis revealed cold temperature storage is an area along the processing chain that allowed for the proliferation and dominance of certain psychrotrophic spoilage microorganisms in meat and dairy (McHugh et al. 2020, Stellato et al. 2016). Monitoring microbial dynamics to understand the effect of storage temperature on the microbial community has been performed using metagenetics coupled with sensory assessment or culture-dependent methods in sausage and fish, which has resulted in the development of models to infer spoilage dynamics and associations of bacterial species during storage (Benson et al. 2014, Zotta et al. 2019). Modified atmosphere packaging (MAP) is currently used to extend the shelf life of various foods like fresh and processed meat and seafood, fruit and vegetables, but optimization of the gas composition is required to keep the product's quality. In the evaluation of MAP for poultry, Wang et al. (2017) identified a shift in the bacterial community compared to other packaging conditions using metagenomics, and Höll et al. (2020) used metatranscriptomics to monitor the regulation responses of two spoilage bacteria to different atmospheric conditions. Similarly, evaluations of shelf life of fish fillets in MAP and vacuum packaging at low temperatures have been performed with metagenetics and sensory analysis or metabolomics to understand the dynamics of spoilage bacteria over time (Jääskeläinen et al. 2019, Sørensen et al. 2020). The efficacy of cleaning and disinfection has been investigated with metagenetics, with evidence of bacterial diversity and abundance altered after cleaning in dairy and pig facilities (Bridier et al. 2019, Dass et al. 2018). Similarly, RNA-based 16S rRNA sequencing showed current cleaning practices with ozonation were effective and caused shifts in potentially active microbiota in meat-processing plants (Botta et al. 2020). In contrast, sanitation in salmon-processing plants, determined by metagenetics, was found to be inadequate as *Pseudomonas* spp. persisted in biofilms on conveyor belts (Langsrud et al. 2016).

Ultimately, sequencing-based meta-omic approaches have been found to be effective tools in identifying microorganisms along the processing chain, and routine implementation can help to uncover the factors that influence microbial population dynamics (McHugh et al. 2020, Zwirzitz et al. 2020). The numerous studies carried out to date show that there is great potential for the use of meta-omic approaches in tracking microbial communities along the food chain.

## SUMMARY POINTS

1. Microorganisms are important contributors to the quality and safety of a food product and they exist throughout the whole food chain.
2. As microbes exist in communities, it is valuable to study them as such. Meta-omic approaches bypass the need for culturing and isolating microbes and allow for the greater characterisation of microbial communities.
3. Metagenetics and metagenomics are two sequencing-based meta-omic approaches that are already being used in the characterisation of foods, food-associated environments and food processing microbiomes. Although only a few studies have used metatranscriptomics, results show potential in assessing the dynamics of viable microbes along the food chain.
4. The use of sequencing-based meta-omic approaches shows promise in better characterisation of microbiomes along the food chain and would allow for greater understanding of the factors contributing to food safety and quality. However, standardized workflows/pipelines are necessary to allow for data sharing and comparability and widespread adoption at a regulatory and industry level.

## FUTURE ISSUES

1. With increasing adoption of these meta-omic approaches to uncover the microbiome of food and food-related environments, there is a great need for standardized workflows/pipelines for methodology and analysis.
2. Large amounts of data are generated by sequencing. This requires good data management practices and systematic metadata documentation to facilitate data sharing of research outputs. Additionally, bioinformatics expertise for the analysis of the data generated is currently essential to draw accurate and correct interpretations from the sequencing data. Future efforts will need to focus on accurate, automated analytical tools.
3. As substantial parts of the analysis require referencing available databases, the results from sequencing studies are only as good as these databases. Databases are currently compiled mainly from human microbiome studies, as more research has been done in that field, which may result in a bias toward human-related microbes. The ongoing

increase in microbiome studies on food and other fields should correct this imbalance to enable better characterisation of microbiomes.

4. With the further development of assays to overcome the challenges of meta-omic approaches, such as host DNA depletion and the ability to distinguish viable microbes in the microbial community, there will be an even wider application of meta-omic approaches for the characterisation of microbes along the food-processing chain.
5. From metagenomic data, the recovery of MAGs could make way for more single-strain studies that can contribute to a greater understanding of the resident microflora of food environments as well as the strains responsible for fermentation or spoilage in foods. Additionally, increasing the number of studies into the functional properties of microorganisms within food environments using metatranscriptomics or metagenomics with complementary approaches like metabolomics can provide greater insight into the active microorganisms and metabolic pathways involved in processes along the food chain.
6. Portable sequencing devices from ONT have allowed for field/onsite sequencing which has proven to be useful in clinical outbreak investigations and environmental sampling. These portable devices could enable rapid detection of microbiological contaminants or pathogens in food-production or food-processing environments. Although some studies have explored this possibility, further comparisons with other sequencing technologies and platforms are required to determine accuracy and comparability (McHugh et al. 2021, Yang et al. 2020).

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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## TABLES

Table 1. Some classical examples of types of food spoilage that can be caused by different organisms.

| **Spoilage characteristic** | **Spoilage organism in food type** | **References** |
|---|---|---|
| Off-odor and off-flavors | *Pseudomonas* spp., *Carnobacterium* spp., *Serratia* spp., *Leuconostoc* spp. and *Brochothrix thermosphacta* produce off-odors and off-flavors in meat, fish and poultry<br>*Shewanella putrefaciens* causes rancid and sulfurous odors and *Aeromonas* spp. produces a sour flavor in smoked salmon<br>Various Enterobacteriaceae cause off-odors and off-flavors in preserved seafood products<br>*Citrobacter* and *Proteus* have been found to cause off-odors in poultry<br>*Candida* spp. and *Kluyveromyces* spp. cause off-odors and flavors in fermented dairy products. | Blackburn (2006),<br>Stohr et al. (2001),<br>Fleet (2011) |
| Changes in texture | *Pseudomonas* spp. cause meat and poultry to become slimy/mushy due to the action of degradative enzymes<br>LAB can cause poor texture in cheese<br>*Bacillus* spp. are able to cause ropiness in breads and bakery products<br>*Clostridium* spp. and *Bacillus* spp. cause softening in vegetables and fruit<br>*Erwinia* and *Penicillium* spp. cause soft rots in vegetables, leading to a mushy texture.<br>The texture of cheese and yogurts is altered by *Candida* spp. and *Kluyveromyces* spp. | Blackburn (2006),<br>Nychas and Panagou (2011),<br>Fleet (2011) |
| Discolouration | *Pseudomonas fluorescens* is able to cause blue coloration in cheese<br>*Carnobacterium viridans* causes green discoloration in cooked cured sausage | Nogarol et al. (2013),<br>Peirson et al. (2003) |
| Gas formation | *Clostridium* spp. cause gas formation resulting in bloating in canned or vacuum-packed goods and late blowing defects in cheese<br>Enterobacteriaceae is responsible for gas production in salad products<br>*Saccharomyces* causes gassiness in wines<br>Several yeast species cause swelling in juice packets | Petruzzi et al. (2017),<br>Sahu and Bala (2017) |

**FIGURES**

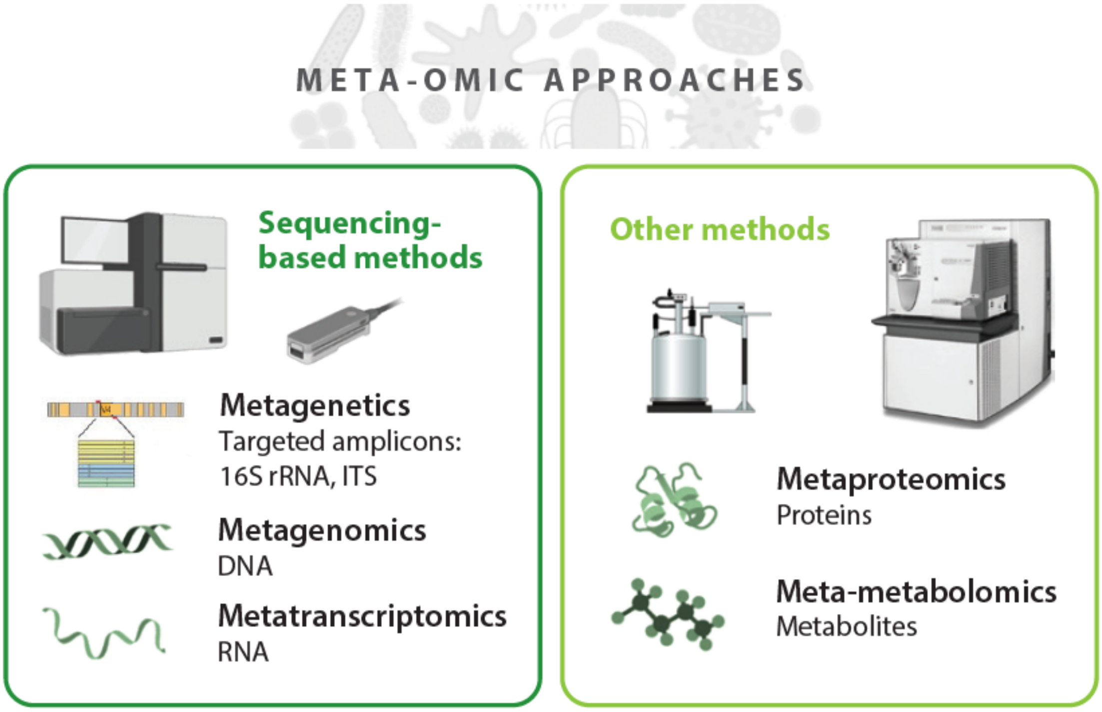


Figure 1. Current meta-omic approaches used in microbiome research. Sequencing-based approached include Metagenetics, Metagenomics and Metatranscriptomics and other community-based methods include Metaproteomics and Meta-metabolomics, which are currently being used in human, environment, and food microbiome studies.

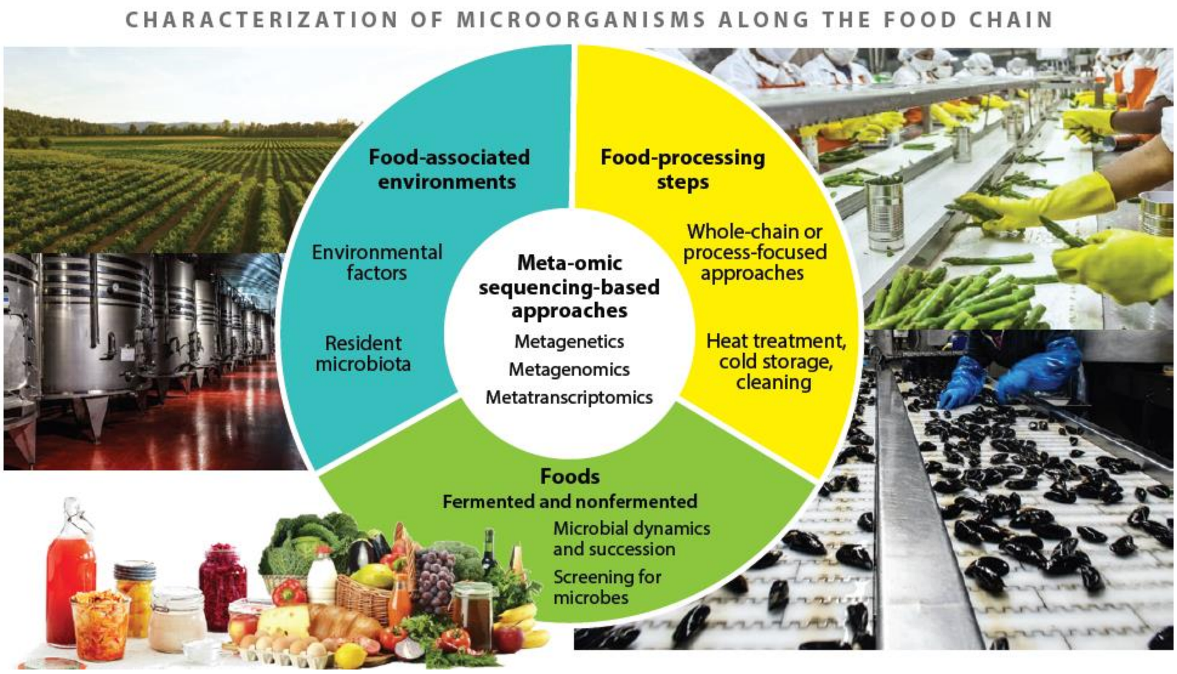


Figure 2. Current applications of meta-omic sequencing-based approaches along the food chain. Metagenetics, Metagenomics and Metatranscriptomics have been used in studies investigating the microbial community of food, food processing steps and food-associated environments.